Effects of di-(2-ethylhexyl) phthalate and four of its metabolites on steroidogenesis in MA-10 cells

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Abstract

Plasticizers aid in processing, and impart flexibility to plastics. Their broad use and tendency to leach out of polymers have rendered them ubiquitous in the environment. Phthalate plasticizers, in particular di-(2-ethylhexyl) phthalate (DEHP), are known to cause male reproductive tract defects in animal models. It has been assumed that DEHP metabolite, mono-(2-ethylhexyl) phthalate (MEHP), is the active compound, however the bioactivity of metabolites such as 2-ethylhexanol, 2-ethylhexanal and 2-ethylhexanoic acid, has not been thoroughly investigated. The aim of this study was to test the effects of these compounds in a mouse Leydig tumour cell line, MA-10 cells. DEHP, MEHP and 2-ethylhexanal decreased cell viability, as well as steroidogenic potential as quantified by an enzyme-linked immunosorbent assay and gene expression analysis. Interestingly, 2-ethylhexanal was the most potent steroidogenic disruptor, which offered an intriguing contribution to the search for the mechanism(s) of phthalate toxicity and raised doubts that MEHP is the single active metabolite.

Résumé

Il est connu que les phtalates utilisés comme plastifiants et plus particulièrement les phtalates di-(2-éthylhexyle) (DEHP) sont à l'origine des malformations de l'appareil reproducteur masculin dans les modèles d'animaux. Ces plastifiants sont essentiels lors de la fabrication des plastiques pour leur donner de la flexibilité. Dus à une utilisation excessive de ces plastifiants et à leur capacité à s'échapper des plastiques existant, ces plastifiants sont maintenant omniprésents dans l'environnement. Dans cette étude, on fait l'hypothèse que le métabolite de DEHP, le phtalate mono-(2-éthylhexyle) (MEHP), est la molécule active, bien qu'il n'existe aucune autre étude concernant les métabolites comme 2-éthylhexanol, 2-éthylhexanal et l'acide 2-éthylhexanoïque. Le but de cette étude est d'évaluer les effets de ces produits chimiques sur une lignée cellulaire tumorale interstitielle du testicule de souris, les cellules MA-10. On a montré que le DEHP, le MEHP et le 2-éthylhexanal ont diminué la viabilité des cellules, aussi bien que la stéroïdogénèse qui a été quantifiée par la méthode ELISA et par l'analyse de l'expression génétique. Contre toute attente, le 2-éthylhexanal s'est avéré le plus puissant acteur dans la dégradation de la stéroïdogénèse, ce qui a ouvert une nouvelle avenue de recherche concernant le ou les mécanisme(s) impliqués dans la toxicité des plastifiants phtalates et a mis en doute le fait que le MEHP est le seul métabolite actif.

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1 Introduction

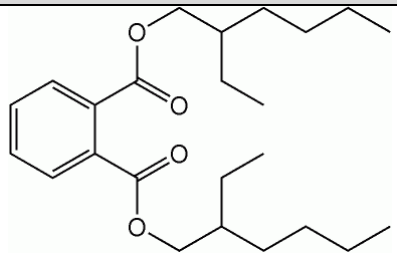
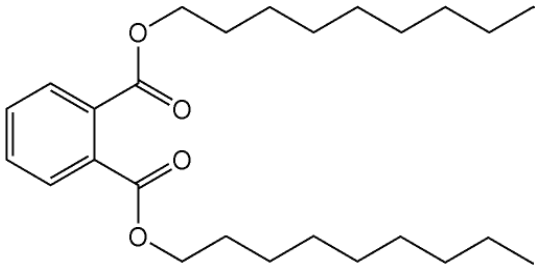
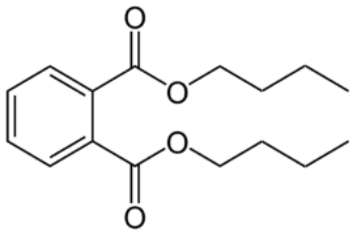
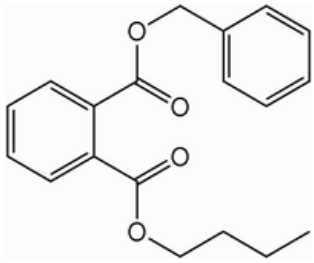
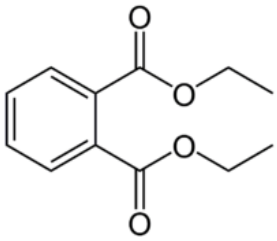
1.1 The Buzz about Phthalates

Rising incidence of male reproductive tract disorders, such as cryptochordism[1] and hypospadias[2], testicular cancer[3,4] and reduced semen quality[5-7] in developed countries has lead to the investigation of certain environmental chemicals as the possible culprits behind these alarming trends[8]. The collection of disorders has been termed testicular dysgenesis syndrome (TDS), and several researchers believe that endocrine disrupting chemicals, such as phthalate plasticizers acting as anti-androgens (disruption of testosterone production), are involved in the etiology of the syndrome[8]. This belief is based on the evidence of some epidemiological studies that have correlated increased phthalate exposure to TDS-like symptoms[1,7], and the almost overwhelming amount of research reporting male reproductive tract malformations in animal models exposed to phthalates[9-20]

Phthalate plasticizers belong to a family of small molecules added to polymers, such as polyvinyl chloride, to improve their flexibility and aid in processing. They are diesters of phthalic acid and are odorless, colourless (or slightly yellowish) oily liquids at ambient temperature. Some common plasticizers and their structures are presented in Table 1 below.

In 2004 it was estimated that phthalate plasticizers were produced on the order of 6 million tons per year worldwide and DEHP accounts for about 50% of total plasticizer production, making it the most common plasticizer in use[21]. Over 80% of plasticizers are used in polyvinyl chloride (PVC), and they can account for 10-60% of a plastic by weight[21]. Some of the uses of plasticizer-enriched plastics include cable insulation, pavements, coatings, imitation leather, lubricants, sealants, food packaging, toys, cosmetics and medical devices[21,22]. In fact, DEHP-enriched PVC is the most common plastic used for medical devices, which leads to high exposure levels[23], as described in greater detail in section 1.3.1. Given the widespread use of these products and the tendency of plasticizers to leach out of plastics[24-26], they have become

Table 1: Some common phthalate plasticizers.

Plasticizer	Structure
Di(2-ethylhexyl) phthalate (DEHP)	
Diisononyl phthalte (DINP)	
Di-n-butyl phthalate (DBP)	
Benzyl butyl phthalate	
Diethyl phthalate	

ubiquitous in the environment, detectable in soil, air, water and even in the urine of the general public[22,27,28].

While evidence of phthalate toxicity has existed for over sixty years, it wasn't until June of 2009 that Canada issued regulations restricting their use[29]. The restriction, part of Canada's Hazardous Products Act, ensures that children's toys and care products imported and sold in Canada do not contain potentially hazardous concentrations of six phthalates (including DEHP) already controlled in the United States and the European Union. Initiatives to develop biodegradable plasticizers that do not have adverse health effects in humans are already underway at McGill University in anticipation of such bans.

Despite this ban, the mechanisms of toxicity of phthalates remains poorly understood. DEHP is rapidly metabolized to 2-ethylhexanol and mono-(2-ethylhexyl) phthalate (MEHP) in the intestine[30], and it has been suggested that MEHP is the active anti-androgenic metabolite responsible for the testicular toxicity of DEHP[16]. This conclusion, however, was reached without adequate investigation of the other metabolites of DEHP. For example, the metabolites of 2-ethylhexanol (2-ethylhexanal and 2-ethylhexanoic acid) have been virtually ignored with respect to their capacity for inducing testicular injury. There is evidence, however, that 2-ethylhexanol and 2-ethylhexanoic acid may be more toxic than the parent compound[28,31]. Assessment of toxicity to aquatic populations has been done in a number of organisms, including Daphnia (*Daphnia magna*) and rainbow trout (*Oncorhynchus mykiss*). The lethal concentrations that caused mortality in 50 % of the population (LC50) for 2-ethylhexanol were 7.7×10^{-4} M and 2.1×10^{-4} M respectively; the LC50 values for 2-ethylhexanoic acid were 9.4×10^{-4} M and 1.2×10^{-3} M respectively[28]. These concentrations are similar to those used in the current study (1×10^{-7}-1×10^{-3} M). As for mammals, the oral rat LD50 (lethal dose that causes mortality in 50 % of the population) of 2-ethylhexanol is 3730 mg/kg[32], while for 2-ethylhexanoic acid it is 3000 mg/kg[33]. The LD50 of the parent compound, however, is an order of magnitude greater, 30 000 mg/kg[34]. These chemicals were detectable in numerous environmental samples (ex. tap water, melted snow, river water and sediment), and in particular the acid is believed to be quite resistant to further degradation[28,35]. While 2-ethylhexanal was not tested in the aquatic

studies due to its high volatility and rapid biodegradation to 2-ethylhexanoic acid in bacterial cultures[28,35], a comparable LD50 of 2600 mg/kg[36] has been reported. Thus the potential for these compounds to exhibit testicular toxicity is a concern. In addition, the molecular targets of DEHP and/or its metabolites have yet to be identified and thus the full mechanism of action of phthalate disruption remains a mystery. Despite the abundance of literature already in existence, further research is required to understand and remedy the impact of phthalates.

1.2 Research Questions

The objective of the study was to evaluate the testicular toxicity of DEHP and its known metabolites *in vitro*. In order to do so, experiments were done with MA-10 cells, a mouse Leydig tumour cell line. A Leydig cell line was chosen as these cells are responsible for the majority of testosterone production in men, and are therefore appropriate for studying anti-androgenic effects. The MA-10 cell line was chosen because it is well established and is capable of responding to gonadotropic stimulation. It should be noted that like most clonal cell lines, MA-10 cells have lost some of their cell specific capacity[37]. Specifically, these cells are not capable of completing all the steps involved in steroidogenesis (production of testosterone). They are, however, capable of performing the first few rate limiting steps, and produce progesterone, which is used as a marker for Leydig cell function. The effects of DEHP and four of its metabolites (MEHP, 2-ethylhexanol, 2-ethylhexanal and 2-ethylhexanoic acid) on this cell line were explored. The research questions investigated were:

- Do DEHP and/or its metabolites affect MA-10 cell viability?
- Do DEHP and/or its metabolites affect MA-10 steroidogenesis?
- Do DEHP and/or its metabolites affect the gene expression of enzymes known to be involved in steroidogenesis?

In answering these questions, this study aimed to test the long standing hypothesis that MEHP is the active metabolite of DEHP. It will also provide an *in vitro* basis for comparison for new plasticizers being developed at McGill

and contributed to the search for the mechanism of action of DEHP in inducing testicular injury.

1.3 Literature Review

1.3.1 Exposure to Phthalates

Given the toxicity of phthalates in rodents and their ubiquity in the environment, many studies investigating the exposure of humans to phthalates have been conducted. Studies of randomly selected subjects have generally shown relatively low levels of phthalate exposure. In 2000, Bount *et al*.[38] completed a study investigating the urinary levels of the monoester metabolites of seven common plasticizers in the general population. The metabolites were used as biomarkers for exposure as the parent compounds are ubiquitous, and thus the measurements subject to contamination. The concentrations of the monoesters were measured in an American reference population and used to estimate the daily exposure levels using the method of Kohn *et al*.[39]; the results are presented in Table 2 below. The table also includes the daily exposure levels to phthalates of the general German population based on urinary concentrations measured by Wittassek *et al*[40]. One final data set is presented as compiled by the Centers for Disease Control (CDC) in the Third National Report on Human Exposure to Environmental Chemicals[41] from nearly 2800 US participants in the National Health and Nutrition Examination Survey administered by the National Center for Health Statistics between 2001 and 2002. The samples were randomly selected to be representative of the US population over the age of 6, and the urinary concentrations converted to daily exposure estimates by the method of Kohn *et al*[39]. Included in the last column of the table is an oral reference dose (RfD) provided by the US Environmental Protection Acgency's (EPA) Integrated Risk Information System (IRIS) for several of the plasticizers. The RfD is a daily dose that is "likely to be without an appreciable risk of deleterious effects during a lifetime" of a human, even the most sensitive[42].

Table 2: Exposure estimates of the general American and German populations to phthalate plasticizers based on urinary concentrations of the monoester metabolites. Values are in µg/kg/day.

Diester	Mean concentration: Blount *et al*.[38]	Mean exposure estimate: Wittassek *et al*.[40]	Mean exposure estimate: CDC[41]	RfD: EPA[42]
Di-ethyl phthalate	14	n/a	6.2	800
Di-n-butyl phthalate	0.92	1.9	0.66	100
n-Butyl benzyl phthalate	1.8	0.2	0.68	200
Dicyclohexyl phthalate	0.076	n/a	< Limit of detection	n/a
Di-2-ethylhexyl phthalate	0.80	2.4	1.1	20
Di-n-octyl phthalate	0.56	n/a	1.1	n/a
Di-i-nonyl phthalate	0.14	0.4	< Limit of detection	n/a

As seen in the table above, most phthalates were several orders of magnitude below the EPA's RfD. DEHP exposure ranged from 0.8-2.4 µg/kg/day for the general public in the US and Germany, which is also below the EPA's oral RfD of 20 µg/kg/day, however only by a single order of magnitude. A study performed by Meek *et al*. measured the exposure of the Canadian population to DEHP through various means (ambient air, indoor air, drinking water, food, etc.) and found significantly higher exposure estimates, varying from 5.8 µg/kg/day for the adult population, up to 19 µg/kg/day for the 0.5-4 year old age group[43]. Although these levels are still acceptable according to EPA standards, one should consider that the EPA's RfD is based on exposure to a single phthalate, and that the general population is clearly exposed to a collection of phthalates, such that the cumulative effects of plasticizers may be

a concern, though research in this area is limited. Moreover, these compounds are known to be further metabolized and therefore urine levels may be a gross underestimation.

While the general population is exposed to relatively low levels of DEHP, certain subsets of the population are exposed to significantly higher levels. These subsets include those with high occupational exposure and those requiring frequent medical attention. Some adverse effects have been associated with occupational exposure[44-46], such as a six-fold increase in the risk of seminoma, a type of testicular cancer, in plastics workers exposed to PVC containing phthalates[46].

Although high levels of DEHP have been measured in humans after medical treatments since the seventies[25,47-55], few long-term studies in humans have been completed to evaluate the risks of DEHP exposure from medical devices. In short term studies, doses of DEHP as high as 140 mg/kg/day have been detected in infants after extracorporeal oxygenation[53]. Other alarmingly high doses have been measured as a result of hemodialysis, blood transfusion and cardiopulmonary bypass as presented in Table 3 below. Given the effects of DEHP on the male reproductive tract in rodents, exposure of infants or pregnant women to such high levels of the plasticizer is a concern.

Table 3: Exposure of medical patients to plasticizers following various treatments. Adapted from Huber *et al*[22].

Treatment	Time period	Dose (µg/kg)	References
Hemodialysis	One session	10-7,200	47,49,51,53
Blood transfusion in adults	Treatment period	200-8,500	48,50
Blood transfusion in newborns	Treatment period	500-4,200	52,55
Extracorporeal oxygenation in infants	Treatment period	42,000-140,000	53
Cardiopulmonary bypass	Treatment day	30-2,400	25,54

1.3.2 Steroidogenesis

Disruption of steroidogenesis by phthalates is suspected to be the cause of many of the male reproductive tract abnormalities reported in animal models[15,56]. One steroidogenic process is sex steroid biosynthesis, which is the process that converts cholesterol to sex steroid hormones, such as testosterone. This occurs almost exclusively in the Leydig cell, an interstitial testicular cell phenotype. Steroid hormone biosynthesis as regulated by trophic hormones can be classed into two categories; "chronic" steroidogenesis, which continuously produces low levels of steroids over a long period of time, and "acute" synthesis, which results in large increases in steroid production and occurs within minutes. The current study investigates only the latter process, and so only acute steroidogenesis will be discussed. Steroidogenesis in Leydig cells is triggered by the binding of luteinizing hormone (LH) to its receptor on the Leydig cell membrane. This induces the production of cyclic adenosine monophosphate (cAMP), an intracellular signaling molecule, from adenosine

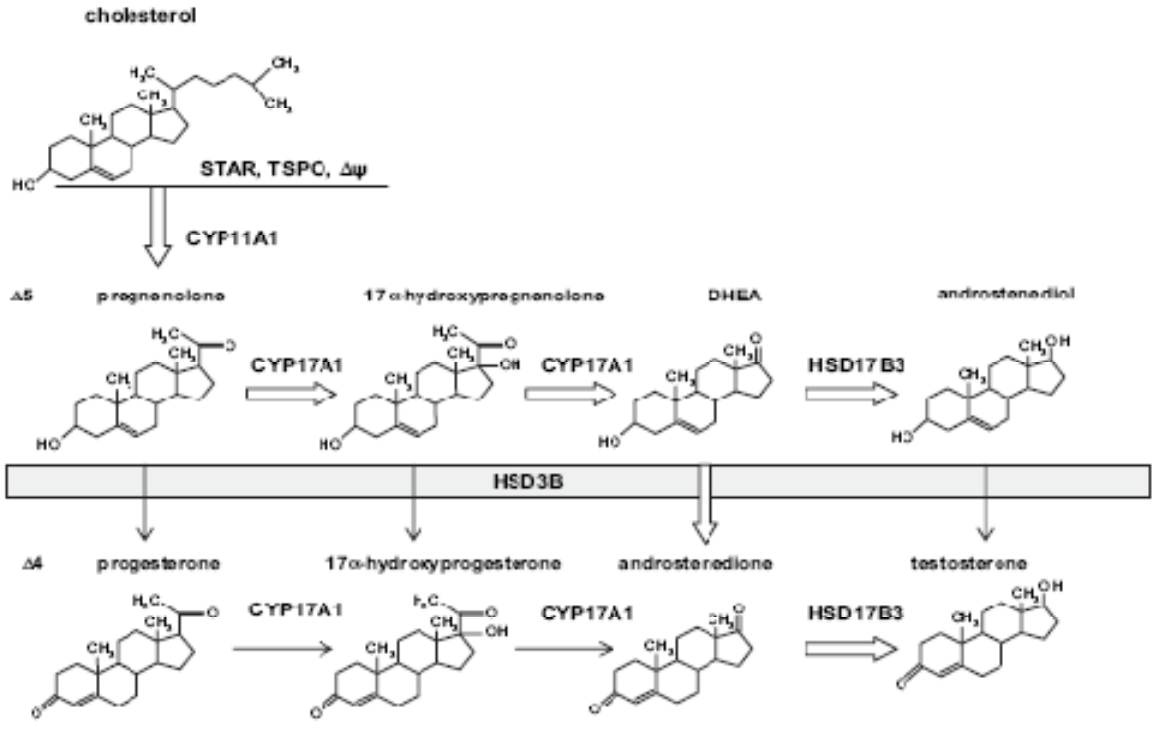


Figure 1: Steroidogenic pathway58. The Δ4 pathway is active in rodents and the Δ5 pathway is active in humans.

triphosphate (ATP). Protein kinase A (PKA) synthesis is catalyzed by cAMP and works with several other enzymes to transport cholesterol from the cytoplasm to the inner mitochondrial membrane. The conversion of cholesterol to testosterone is outlined in Figure 1 above.

These transport enzymes include the steroidogenic acute regulatory protein (StAR), the outer mitochondrial membrane translocator protein (TSPO), formerly known as the peripheral-type benzodiazepine receptor (PBR), and several others[57,58]. Once the cholesterol has passed into the mitochondria, the inner-mitochondrial membrane bound cytochrome P450 side chain cleavage (CYP11A1 or P450scc) enzyme converts it to pregnenolone. Pregnenolone is then transferred to the endoplasmic reticulum where the remaining enzymatic steps occur. The conversion of pregnenolone to testosterone involves a number of intermediates and may proceed via two different routes, called the Δ^4 and Δ^5 pathways. It is the latter pathway that is active in humans; however the Δ^4 is more common in rodents. In either case, the same enzymes are involved.

The Δ^4 pathway begins by the conversion of pregnenolone to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase/ Δ^5-Δ^4 isomerase (HSD3B). This is where the process ends in MA-10 cells[37]. In normal cells, the endoplasmic reticulum membrane bound enzyme cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17A1) then converts progesterone to 17α-hydroxyprogesterone and then androstenedione. Finally, androstenedione is reduced to testosterone by the enzyme 17β-hydroxysteroid dehydrogenase type III (HSD17BIII). In the Δ^5 pathway, CYP17A1 first converts pregnenolone to 17α-hydroxypregnenolone and then dehydroepidandrosterone (DHEA), which is subsequently acted on by 3β-HSD to form androstenedione. Lastly, HSD17BIII converts androstenedione to testosterone[58].

In this study, the gene expression of three key proteins is investigated. The proteins StAR, TSPO and CYP11A1 were chosen due to their involvement in the first two steps of steroidogenesis. The transport of cholesterol from the outer to the inner mitochondrial membrane is the rate limiting step of acute steroid biosynthesis, since simple diffusion of hydrophobic cholesterol across

the aqueous intermembrane space is very slow. Work on the regulation of steroid hormone biosynthesis in the 60s, 70s and 80s revealed that this step is sensitive to inhibitors of protein synthesis, and thus de novo protein synthesis is required for protein transport across the mitochondrial membrane[59]. The search for the proteins and their mechanisms of action involved in this important transport step continues today.

One of the first proteins to be identified as sensitive to acute stimulation and involved in the transport of cholesterol across the mitochondrial membrane was the StAR protein[60]. This protein was identified in cells in two forms; the immature 37-kDa StAR protein containing a mitochondrial leader sequence that directs the protein to mitochondria, where the sequence is cleaved yielding a mature 30-kDa mitochondrial matrix StAR protein. Surprisingly, the final 30-kDa StAR protein is steroidogenically inactive, and it is the 37-kDa immature protein that is responsible for protein transport, providing the first example of a mitochondrial protein being active in an immature state. It is this property that allows for the rapid initiation and termination of acute steroidogenesis[61].

The mechanism of action of StAR protein is not yet fully understood, however direct interaction of the protein with cholesterol is thought to be involved due to the cholesterol binding abilities of StAR[62] and a correlation between cholesterol binding and StAR activity[63]. It is also understood that StAR does not act alone, but is involved in a macromolecular protein complex that also contains TSPO, PAP7, a TSPO-associated protein and the regulatory subunit Iα of protein kinase A (PKARIα)[64].

The first signs that TSPO was involved in steroidogenesis were the observations that the protein is abundant in steroidogenic cells and that it is associated primarily with the outer mitochondrial membrane[65]. Further evidence emerged when Papadopoulos *et al*. showed that several TSPO ligands could increase pregnenelone production to levels in agreement with their binding affinities in MA-10 and primary Leydig cells[66], and that such increases were the result of enhanced cholesterol transport from the outer to the inner mitochondrial membrane[67].

Although the exact mechanism of action of the proteins remains uncertain, one suggestion is that TSPO and another associated protein, the voltage-dependant anion channel (VDAC), form a pore in the mitochondrial membrane through which the cholesterol can pass and that StAR confers steroid specificity to the complex[68]. Later, Hauet *et al*. identified the PAP7 protein and were the first to suggest that it may act by targeting PKARIα to TSPO so that phosphorylation can occur[57]. Finally, Lui *et al*. confirmed the presence of a StAR-PKARIα-PAP7-TSPO complex localized in the mitochondria and proposed that PAP7 links TSPO to PKARIα, conferring cAMP responsiveness to the complex, which then associates with StAR protein, providing its cholesterol transport abilities[64].

The final protein of interest is cytochrome P450 side chain cleavage (CYP11A1) as it is responsible for the conversion of cholesterol to pregnenolone and is considered the rate limiting enzymatic step[69].

1.3.3 Metabolism of DEHP

The first step in the metabolism of DEHP is the enzymatic hydrolysis of the diester by lipases and esterases (phase I hydrolysis) into its primary metabolites, the monoester phthalate MEHP and 2-ethylhexanol, which occurs primarily in the intestine and liver [30,70]. It should be noted that a study investigating the distribution of DEHP or its metabolites in marmosets showed that the greatest accumulation of orally administered plasticizer occurred in the testes 7 days after exposure[71]. Following hydrolysis, MEHP and 2-ethylhexanol then follow separate metabolic pathways. Futher hydrolysis of MEHP to phthalic acid only occurs to a minimal extent[30,72]. Instead it undergoes several other transformations; hydroxylation and oxidation (see Figure 2 below), followed by phase II conjugation, often by the enzyme uridine 5'-diphosphoglucuronyl transferase, to the glucuronide conjugates, which are easily excreted in urine[73]. In fact, approximately 80% of urinary metabolites of DEHP are in the glucuronide conjugated form[74]. Some of the metabolites commonly used as urinary biomarkers of human exposure to DEHP include (in

their unconjugated forms): mono-(2-ethyl-5-hydroxyhexyl)phthalate (5OH-MEHP), mono-(2-ethyl-5-oxohexyl)phthalate (5oxo-MEHP), mono-(2-ethyl-5-carboxypentyl)phthalate (5cx-MEPP) and mono-[2-(carboxymethyl)hexyl]phthalate (2cx-MMHP)[75,76].

2-ethylhexanol on the other hand is first converted to the aldehyde 2-ethylhexanal by the alcohol dehydrogenase enzyme, and then to 2-ethylhexanoic acid by the aldehyde dehydrogenase enzyme[77].

While no experiments investigating the metabolism of DEHP in MA-10 cells have been done, preliminary experiments in human hepatocellular carcinoma (HepG2) cells have confirmed the breakdown of DEHP into 2-ethylhexanol by these cells. However further breakdown of this compound was not observed due to the lack of alcohol dehydrogenase expression in this cell line. Thus exposure of these cells to 2-ethylhexanal and the resultant accumulation of 2-ethylhexanoic acid confirmed the last step in this metabolic pathway *in vitro*[78].

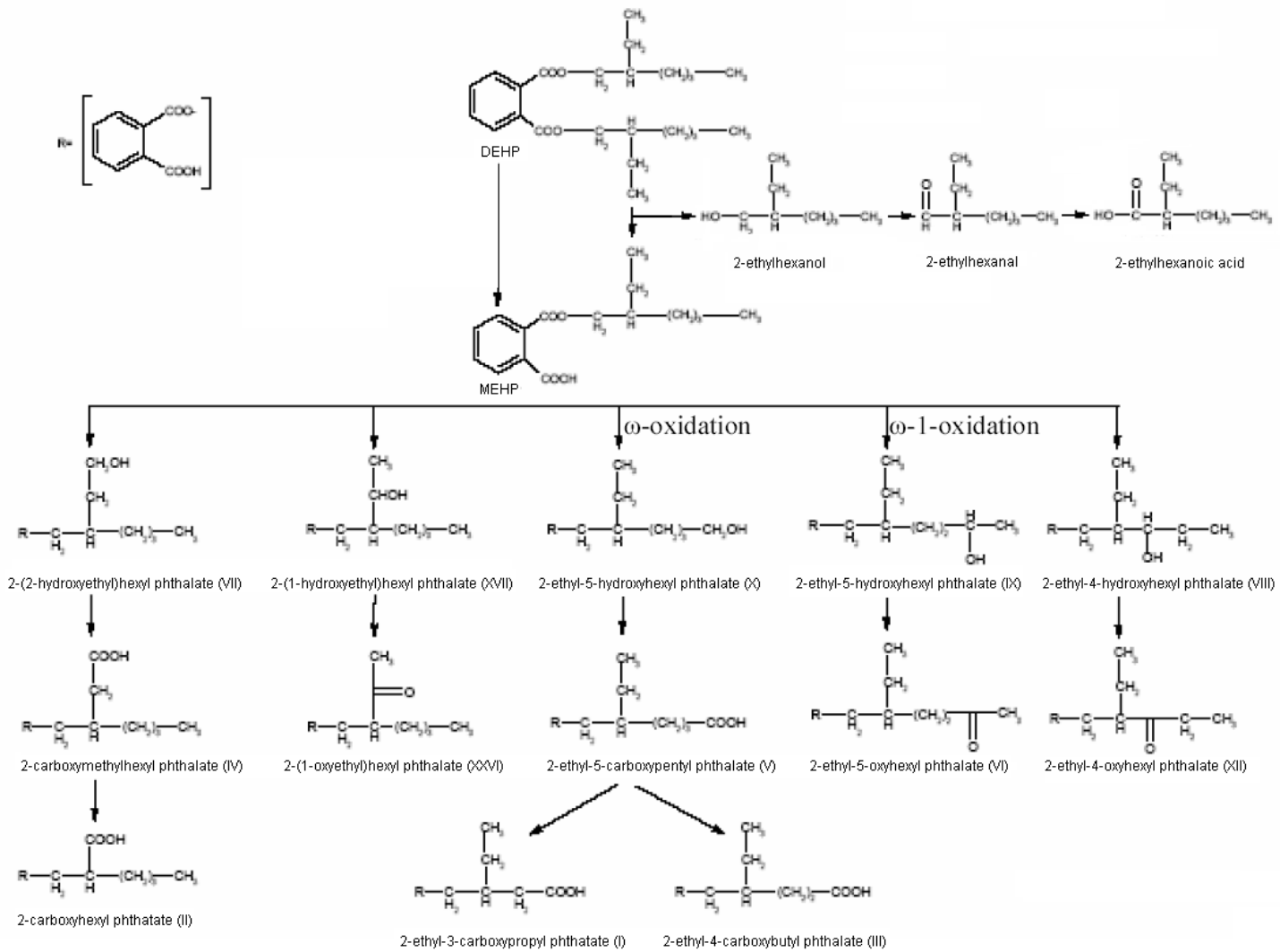


Figure 2: Some metabolic products of DEHP as detected in rat urine, adapted from Albro *et al*[72].

1.3.4 Effects of Plasticizers

Concerns regarding the potential toxicity of plasticizers arose as early as 1945[9], when Shaffer *et al*. showed the first evidence of testicular toxicity in rats orally exposed to DEHP, with tubular atrophy and degeneration in the testis at doses equivalent to 1.6 g/kg/day and higher. In the following three decades, a number of groups studied phthalates as a result of these observations. Relatively high LD50 values in a number of species led researchers to conclude that the use of phthalate esters as plasticizers was justified, and did not pose a significant health risk[79-82].

It was not until 1977 that interest in phthalates was renewed, when Gray *et al*. exposed male and female rats orally to DEHP for 17 weeks. They found that liver weights were reduced, and that in male rats, the testis weight was significantly reduced. In addition, spermatogenesis ceased and severe atrophy of seminiferous tubules occurred[83]. This sparked a cascade of studies investigating the effects of DEHP in multiple organs. Adverse effects have also been reported in the kidneys, lungs and heart[23], however the liver and testes were the main organs of concern. DEHP is known to cause a number of hepatocellular abnormalities, including increased number of peroxisomes, increased fatty acid metabolism, cell proliferation, apoptosis, and a number of other cellular events, all culminating in the induction of hepatic tumours. The relevance of rodent data to humans is still being questioned, however, as human cell cultures have proven to be much less sensitive to DEHP, or simply lack the signaling pathways implicated in rodent hepatic carcinomas[84].

The study performed by Gray *et al*.[83] also directed research towards the Sertoli cell as the target of phthalate induced testicular injury as these cells are part of the seminiferous tubules and are responsible for nurturing sperm cells throughout the process of spermatogenesis[85]. In 1982, Foster *et al*. contributed to the evidence that Sertoli cells were the main target of phthalates with a study in which adult rats were exposed to a single, high dose of di-n-pentyl phthalate (DPP). Vacuolation of the Sertoli cell cytoplasm was the first visible sign of

testicular injury only 6 hours after exposure, followed by degradation of Sertoli cell mitochondria and depletion of zinc in spermatids 24 hours after exposure[10]. Other groups working with MEHP-treated cultured Sertoli cells noted a decrease in follicle stimulating hormone (FSH) induced cyclic adenosine monophosphate (cAMP) production and postulated that this might be the mechanism of testicular injury[11,86].

A shift in focus occurred in the mid-nineties however when developmental abnormalities in the male reproductive tract following in utero/perinatal exposure to phthalates were reported[12,13,87]. These effects included reduced anogenital distance, epididymal dysgenesis, hypospadias, chryptorchidism, Leydig cell hyperplasias and small testis. These abnormalities in androgen-dependent development suggested an antiandrogenic mechanism of injury. A great deal of interest was invested in exposure to phthalate plasticizers during key male reproductive development windows.

Suspicions that phthalate esters might be endocrine disruptors sparked a number of studies that investigated the ability of phthalates to disrupt testosterone production by Leydig cells[15,56]. In 2000, Parks *et al*. showed that treatment of pregnant dams with DEHP caused a marked decrease in testosterone production in fetal and neonatal male rats. The group also showed that neither DEHP nor MEHP exhibited binding affinity for the androgen receptor (AR), and thus the mechanism of action could not be AR agonism or antagonism[56].

Subsequently, exploration of the potential of phthalates to disrupt steroidogenesis by decreasing the expression of steroidogenic enzymes was initiated (see section 1.3.2 for description of these enzymes). Several groups have shown that the expression of some of the key enzymes involved in steroidogenesis are affected by phthalate exposure, including the steroidogenic acute regulatory protein (StAR), the outer-mitochondrial membrane translocator protein (TSPO, also known as the peripheral benzodiazepine receptor, or PBR) and cytochrome P450 side chain cleavage (P450scc)[14,17,19]. Today, the focus of study is elucidating the mode of action of phthalates in

inducing testicular injury. A number of hypotheses exist, including the involvement of the peroxisome proliferator activated receptor (PPAR), a nuclear receptor[17], oxidative stress[88] and the thyroid hormone receptor alpha 1 (TRα1)[89]. These hypotheses will be presented in greater detail in the discussion (section 4.5 Mechanism of Action).

1.3.5 Summary

In summary, the ubiquity of DEHP, rising incidences of TDS in the human population and its long history of testicular toxicity have resulted in its ban from children's toys and products in the US, the EU and now Canada. The need for alternative plasticizers and the understanding of how DEHP exerts its testicular effects is reaching a critical point. This study tests the hypothesis that MEHP is the active metabolite of DEHP by evaluating the effects of DEHP, MEHP, 2-ethylhexanol, 2-ethylhexanal and 2-ethylhexanoic acid in MA-10 cells, a steroidogencially active cell line. In doing so, an *in vitro* basis for comparison of future plasticizers in development at McGill is supplied, and a contribution to the search for the mechanism of testicular toxicity of DEHP is made.

2 Materials and Methods

2.1 Chemicals

A list of chemicals and suppliers is included in Appendix I. Biohazard approval is included in Appendix II. Chemicals intended for use in cell culture were sterilized by autoclave at 121^{0}C for 30 minutes, or filtered with Millex®GV 0.22 μm filter units (Millipore). DEHP and its metabolites were purchased from Sigma-Aldrich, except for MEHP which was purchased from Crescent Chemical Company.

2.2 Cell Culture

MA-10 mouse Leydig tumor cells were a generous gift of Dr. Mario Ascoli, via Dr. Tremblay (Université Laval). The cells were cultured in Waymouth's MB 752/1 (1X) medium supplemented with 15 % horse serum, 20 mM HEPES and 0.25 % penicillin-streptomycin (Gibco) at 37^{0}C in 5% CO2 (Thermo Electron Corporation, Model Forma Series II Incubator). The media was changed every second day and the cultures were split 1:3 every 3-4 days. Flasks were pre-treated with 0.1 % gelatin from porcine skin (Sigma-Aldrich, G2500) to improve cell adhesion. This cell line did not adhere very strongly, such that all handling of the cells was performed very carefully so as not to detach the cells. Details of the cell culturing protocols can be found in Appendix III.

2.3 Cell Viability Assay

Pass 13 MA-10 cells were exposed to DEHP and four known metabolites of biodegradation in static culture and the effect on cell viability was assessed using an MTT colorimetric assay that measures the metabolic activity of the cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT (Sigma-Aldrich, M5655) is a compound that is rapidly metabolized by healthy

cells, producing purple formazan crystals that can be easily quantified by spectrometry.

2.3.1 Sample Preparation

A confluent flask of MA-10 cells was trypsinized and seeded in a 96-well plate pre-treated with gelatin (Sigma-Aldrich, G2500) at a concentration of 5000 cells per well. Extra wells were seeded at this concentration, to serve as controls, and three wells were filled with media alone to serve as blanks. A standard curve was prepared using cell suspensions of seven different concentrations (1000; 2000; 4000; 5000; 6000; 8000; 10 000 cells/well). Each standard, sample, control and blank was prepared in triplicate. The cells were allowed to attach overnight at 37^0C in 5% CO_2.

The following day, the media was removed from the sample wells and 100 µL of media with 0.3 v/v % dimethylsulfoxide (DMSO) and various concentrations (1x10-3; 1x10-4; 1x10-5; 1x10-6 M) of DEHP, MEHP, 2-ethylhexanol, 2-ethylhexanal and 2-ethylhexanoic acid were added to the wells. Loading was randomized in the plate. Media from the standard, control and blank well was removed and replaced with 100 µL of media with 0.3 v/v % DMSO. As two time points were investigated, the plates were incubated for either 24 or 48 hours 37^0C in 5% CO2.

2.3.2 MTT Assay

At the end of the exposure period, the plate was removed and 110 µL of phenol red free (transparent) media with 1.1 mM MTT was added, according to the manufacturer's protocol. The plate was then incubated at 37^0C in 5% CO2 for 4 hours. The formation of formazan crystals was confirmed under a light microscope after the incubation period, and the crystals were solubilized by adding 100 µL of SDS-HCl detergent and incubating the plate for 18 hours at 37^0C in 5% CO2. The optical density, or absorbance, of each well was measured using a Benchmark Plus™ microplate spectrophotometer (Bio-Rad) at 570 nm. The absorbance of wells with cells treated with the aforementioned

compounds was normalized to the absorbance of the control wells to assess cell viability.

2.4 Effects on Steroidogenesis

MA-10 cells are not capable of producing the enzymes required for the production of testosterone, but do produce progesterone, an intermediate hormone. Therefore, progesterone was used as a marker for testosterone production. The progesterone produced as the result of acute stimulation with human chorionic gonadotropin (hCG, 0.5 nM, ≥ 12 000 IU/mg, Calbiochem, 869031) was measured using a competitive binding enzyme-linked immunosorbent assay (ELISA, Fitzgerald Industries International, Inc., 55R-RE52231).

2.4.1 Sample Preparation

Samples were prepared as described for the cell viability experiments in ten 6-well plates at a concentration of 40 000 cells per well. The following day, the media was removed from the sample wells and 1.6 mL of media with 0.3 v/v % dimethylsulfoxide (DMSO) and various concentrations of DEHP, MEHP, 2-ethylhexanol, 2-ethylhexanal and 2-ethylhexanoic acid was added to the wells in a randomized pattern. Media from the control wells was removed and replaced with 1.6 mL of media with 0.3 v/v % DMSO. The plates were incubated for 24 hours at 37^0C in 5% CO2.

After 24 hours, the media was removed and the wells washed with phosphate buffered saline (PBS) to remove any excess compound. The cells were then stimulated with 1.6 mL of media with 0.5 nM hCG per well for 4 hours at 37^0C in 5% CO2. Only the negative control wells were not stimulated, instead the media was replaced with fresh media. The supernatant was then collected and stored at -20^0C for analysis within two weeks. The cells were washed twice more with PBS and saved for total protein analysis.

2.4.2 Progesterone Measurement

The ELISA kit and samples were allowed to reach room temperature before beginning the protocol provided by the manufacturer. Progesterone standards (0; 0.3; 1.25; 2.5; 5; 15; 40 ng/mL progesterone) from the kit, samples, controls and blanks were added at a volume of 25 µL to the microtiter wells and the plate was incubated at room temperature for 5 minutes. The enzyme conjugate solution supplied in the kit was then added to each well and the plate was incubated at room temperature for one hour. After one hour, the wells were thoroughly rinsed with the wash solution provided and the enzyme substrate solution was added. The reaction was allowed to proceed for 15 minutes, at which time the stop solution was added to terminate the reaction and arrest the colour change. The optical density of each well was measured using a Benchmark Plus™ microplate spectrophotometer at 450 nm. As the assay is based on the principle of competitive binding, a 4 parameter logistic (4PL) curve was used to fit the standard curve and thus calculate the progesterone concentrations in the samples.

2.4.3 Total Protein Measurement

Owing to the possible variations in the number of cells in each well, the progesterone production was normalized to total protein. A Bicinchoninic Acid (BCA™) Protein Assay Kit (Thermo Scientific, 23227) used to quantify the concentration of protein per well. The basis of this assay is the biuret reaction, wherein Cu+2 is reduced to Cu+1 by protein in an alkaline medium. The Cu+1 ion is then detected by a colour change that results from the chelation of two BCA molecules with one Cu+1 ion.

Cells were treated in 6 well plates as described in the sample preparation section above. They were then lysed by adding 400 µL of RIPA lysis buffer to each well, and shaken for 10 minutes. Cell lysis was confirmed visually using a light microscope (Leica DM IL). During the lysis period, a standard curve was prepared from a stock solution at 2 mg protein/mL of bovine serum albumin (BSA) supplied in the assay kit. The standard concentrations used were 0, 25, 50, 75, 100, 150 and 200 µg protein /mL. Also during this period, the

"Working Reagent" (WR) was prepared by mixing 50 parts of BCA™ Reagent A (containing BCA) with 1 part BCA™ Reagent B (containing cupric sulfate, the source of Cu+2 ions). Once the lysis was complete, 25 µg of each standard, sample and control was transferred to a 96 well plate (triplicates of each) and 200 µL of WR was added to each well. The 96-well plate was then incubated at 37 ^{0}C for 2 hours. Following the incubation period, the absorbance of each well was measured using a Benchmark Plus™ microplate spectrophotometer at 562 nm, and the total protein concentration in each sample was calculated using the linear standard curve.

2.5 Gene Expression Analysis

The ability of the compounds to alter gene expression, in particular, the genes involved in the acute production of progesterone was investigated by quantitative real-time polymerase chain reaction (qRT-PCR). These include *Star*, the gene encoding the steroidogeneic acute regulatory (StAR) protein, *Cyp11a1*, the gene encoding the cytochrome P450 side chain cleavage (P450scc) enzyme and *Tspo*, the gene encoding the outer mitochondrial membrane translocator protein (TSPO). The gene encoding 18S ribosomal RNA (*18S*) was used as a housekeeping gene. SYBR green was used as a detector to quantify the mRNA of each gene.

2.5.1 Sample Preparation

Samples were prepared as previously described in section 2.3.1 in four 6-well plates at a concentration of 320 000 cells per well. Following 0.5 nM hCG stimulation, the cells were washed with PBS before RNA extraction.

2.5.2 RNA Extraction

The Qiagen RNeasy Mini Kit (74104) was used to extract RNA according to the manufacturer's protocol. The basis of the technique is the selective binding properties of RNA to a silica based membrane. Microcentrifuge tubes are packed with said membrane, to which the homogenized cell lysate is added. Centrifugal force is used to pull the lysate through the membrane. Enrichment

of mRNA occurs since RNA molecules smaller than 200 nucleotides (small rRNAs and tRNAs) pass through the membrane. The purified mRNA is then eluted with water.

Cells in the 6-well plates prepared as described above were lysed by adding 350 µL of the Buffer RLT provided and the DNA sheared by passing the lysate through a blunt 20 ½ gauge needle. Further homogenization was achieved using Qiagen QIAshredder (79654) spin columns. Following homogenization, the lysate was mixed with an equal volume of 70% ethanol to enhance the binding of RNA to the silica membrane in the RNeasy spin columns, and the ethanol-lysate solution was added to the RNeasy spin columns provided by the kit. The columns were centrifuged at 8000 x g for 30 seconds, and the flow-through was discarded. The membrane was rinsed with 350 µL of Buffer RW1 by the same method and the flow-through discarded.

In order to ensure that no genomic DNA is carried over, a digestion with the Qiagen RNase-free DNase set (79254) was done. This involved mixing 70 µL of Buffer RDD provided in the kit with 10 µL of reconstituted DNase I enzyme solution, and adding the mix to each column for 15 minutes at room temperature.

Following the digestion, the column was rinsed once with 350 µL of Buffer RW1, then twice with 500 µL of Buffer RPE. The column was then spun down without any buffer to dry it, and placed in a new RNA collection tube. To elute the RNA, 40 µL of nuclease-free water was added to the column and it was spun down, and the eluate passed through the column a second time to collect any residual RNA. The RNA was stored at -80^{0}C until it was quantified and reverse transcribed into cDNA.

2.5.3 RNA Quantification

A Jenway Genova MK3 Life Science Analyser UV/Vis spectrophotometer was used to quantify the extracted RNA. The principle is based on the fact that DNA and RNA absorb ultra violet light, with an absorption maximum at 260

nm. Based on the Beer Lambert Law, the concentration can be calculated as follows;

$$C(\mu g / mL) = A \times dilution\ \ factor \times 40\ \mu g / mL$$

As proteins tend to absorb at 280 nm, the ratio of absorbance at 260 nm to 280 nm was used to asses the degree of amino acid contamination. A ratio between 1.8 and 2 is considered sufficiently pure for qRT-PCR, with 2 being the ideal ratio.

The ultraviolet source was turned on to warm up for 20 minutes before use, while the RNA samples thawed on ice. The RNA was diluted by adding 5 µL of RNA to 120 µL of deionized water (DW) in a 0.6 mL RNase-free microcentrifuge tube for a dilution factor of 25. The cuvette was rinsed several times with DW and dried with compressed air. The spectrophotometer was set on DNA/RNA mode and the appropriate settings entered. DW was added to the cuvette and placed in the spectrophotometer for calibration. The water was discarded, the cuvette dried, the first sampled loaded into the spectrophotometer and the absorbance at 260 nm and 280 nm read and recorded. Between each sample, the cuvette was rinsed several times and a blank read to ensure that no cross-contamination occurred.

2.5.4. Primer Design

Primers were designed using Primer3 v0.4.0[90] available online at http://frodo.wi.mit.edu/. Mouse gene specific primers used to amplify *Star* were 5'-TTGGGCATACTCAACAACCA-3' (forward) and 5'GAAACACCTT GCCCACATC T-3' (reverse); primers for *Cyp11a1* were 5'-CCATCAGATGC AGAGTTTCCAA-3' (forward) and 5'-TGAGAAGAGTATCGACGCATCCT-3' (reverse); primers for *Tspo* were 5'-TGCAGAAACCCTCTTGGCATC-3' (forward) and 5'-TGAAACCTCCCAGC TCTTTCC-3' (reverse); and primers for *18S* were 5'-GCAATTATTCCCCATGAACG-3' (forward) and 5'-GGATG CAGGGATGATGTTCT-3' (reverse). All primers were purchased from Alpha DNA.

2.5.5 Reverse Transcription (RT)

RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (ABI, 4368814). This kit employs a hot start reverse transcriptase enzyme that converts RNA into cDNA with random primers and excess nucleotides provided in the kit.

Briefly, the RT master mix was prepared by mixing the random primers, dNTPs, RNase inhibitor, MultiScribe™ reverse transcriptase, and nuclease-free water in the RT buffer. The mix was then added to 0.2 mL PCR (STARSTEDT, 72 737 002) tubes at a volume of 10 µL, and then 10 µL of sample RNA was added to each tube, without exceeding 2 µg per 20 µL reaction volume. The tubes were loaded into a thermal cycler programmed for reverse transcription and the reaction started. The resulting cDNA was stored at -20^{0}C.

2.5.6 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The ABI 7900HT Fast Real-Time PCR system was used along with Power SYBR® Green Master Mix (ABI, 4367659). PCR master mix was prepared by mixing 5 µL/reaction of SYBR® Green master mix (containing both the SYBR® green dye and the DNA polymerase enzyme) with 1 µL/reaction of each of the forward and reverse primers at the optimum concentrations shown in Table 4 and 1 µL/reaction of nuclease-free water. After gently mixing and briefly spinning down the PCR master mix, 8 µL was added to each well of the 384-well reaction plate (ABI). Subsequently, 2 µL of cDNA diluted to 0.5 ng/µL was added to each well. The standard curve was constructed with points at 0.01; 0.1; 1; 10; and 100 ng cDNA per 10 µL reaction. The plate was sealed with an optical adhesive cover, shaken for 1 minute and centrifuged for 1 minute at 3000 rpm. The plate was then placed in the 7900HT Fast Real-Time PCR system and the samples were amplified over 40 cycles under the conditions listed in Table 5. Exponential amplification usually occurred between cycles 5 and 15 for the housekeeping gene and 15 and 25 for the target genes, yielding C_t values in these ranges. A dissociation curve was performed

at the end of the run to ensure specific amplification of the target gene and rule out the formation of primer-dimers. For each target, the amplification products were run at least once on an agarose gel to confirm the amplification of a single target. The data was processed using the relative quantification method.

Table 4: Optimized primer concentrations

Primer	Optimum concentration in 10 µL reaction (nM)
Star (forward and reverse)	200
Cyp11a1 (forward and reverse)	100
Tspo (forward and reverse)	200
18S (forward and reverse)	200

Table 5: qRT-PCR cycle conditions

Stage	Temperature (°C)	Time	Cycles
DNA polymerase activation	95	20 seconds	Hold
Denature	95	1 second	40
Anneal/Extend	60	20 seconds	40
Dissociation curve	95	15 seconds	1
Dissociation curve	60	15 seconds	1
Dissociation curve	95	15 seconds	1
Cool down	40	2 minutes	Hold

2.6 Statistical Analysis

Statistical analysis was performed using Prism 5.0 software (GraphPad Software Inc., San Diego, California) and SYSTAT 12 (SYSTAT Software Inc., Chicago, Illinois). Bar graphs show mean values with error bars representing the standard deviation of the mean. Both one-way and two-way repeated measures Analysis of Variance (ANOVA) were used and Bonferroni post-tests were used to identify significant differences. Differences were considered significant is the P values were less than 0.05.

3 Results

3.1 Cell Viability

The effect of DEHP and its metabolites on the cell viability of MA-10 cells was determined using an MTT assay. MA-10 cells were exposed to the DEHP, MEHP, 2-ethylhexanol, 2-ethylhexanal and 2-ethylhexanoic acid at concentrations varying from $1x10^{-6}$ M to $1x10^{-3}$ M for 24 and 48 hours. The treated samples were normalized to the control sample (media with DMSO at 0.3 v/v %). DMSO was used to solubilize the compounds and had no effect on cell viability at the concentrations used (results not shown). A two-way ANOVA of the data from 5 independent experiments showed that there was no significant effect of the compounds ($P=0.1951$, two-way ANOVA). Concentration did have an effect ($P<0.005$, two-way ANOVA) however there was significant interaction in the data. Only MEHP at the highest concentration ($1x10^{-3}$ M) was significantly different than all other compounds at the same concentration (DEHP $P<0.001$; 2-ethylhexanol $P<0.001$; 2-ethylhexanal $P<0.001$; 2-ethylhexanoic acid $P<0.01$, Bonferroni post test). MEHP reduced the viability of the cells by approximately 30 % compared to the control at this concentration as shown in Figure 3 below.

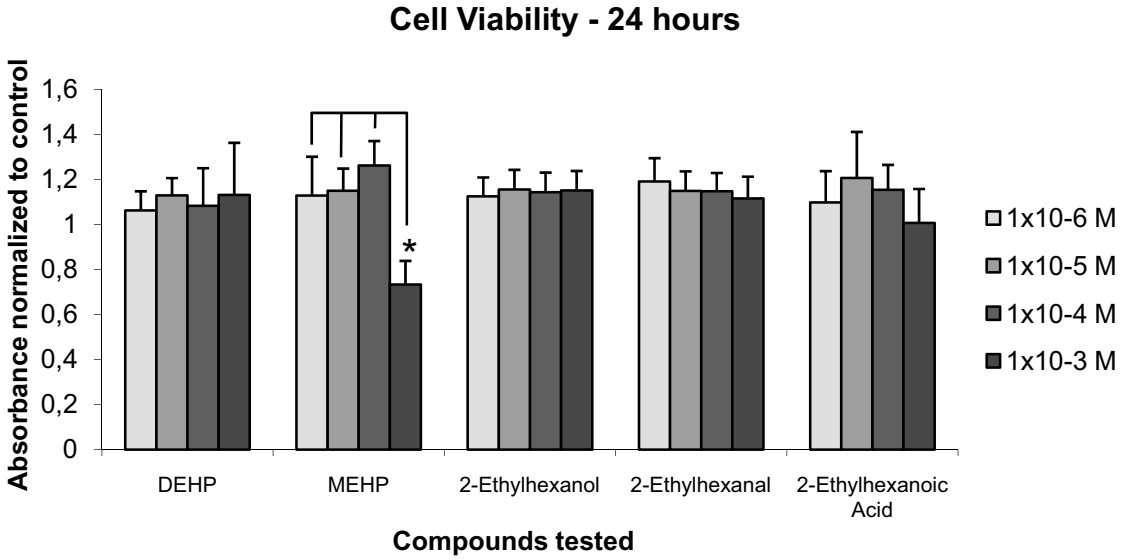


Figure 3: Effect of DEHP and metabolites on cell viability after 24 hours exposure. Only MEHP at the highest concentration ($1x10^{-3}$ M) reduced cell viability significantly. * P<0.05, Bonferonni post test.

After 48 hours of exposure, the parent compound, DEHP, the monoester MEHP and 2-ethylhexanal significantly reduced cell viability (Figure 4). DEHP reduced the viability at the two highest concentrations in a dose dependent manner (>60 % at $1x10^{-3}$ M and >40 % at $1x10^{-4}$ M) while the aldehyde caused a small but significant reduction in cell viability at $1x10^{-3}$ M. As at 24 hours, MEHP exhibited the strongest reduction in cell viability (approximately 85 %) at a concentration of $1x10^{-3}$ M. One other trend that was noticeable but not significant at 48 hours was the apparent reduction in cell viability after exposure to the highest concentration of 2-ethylhexanoic acid.

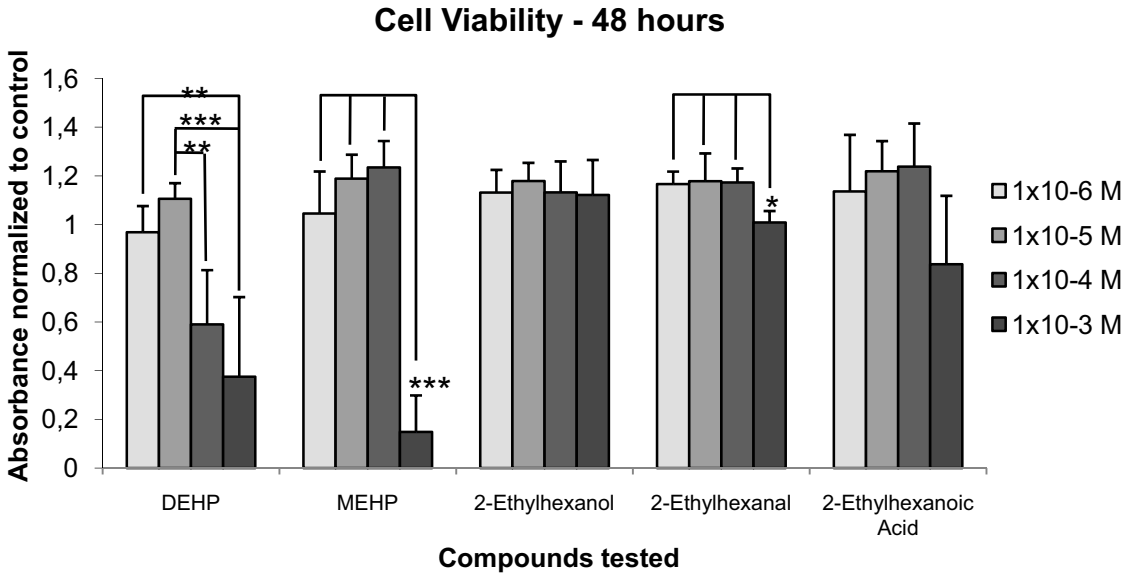


Figure 4 : Effect of DEHP and metabolites on cell viability after 48 hours exposure. MEHP significantly reduced cell viability by 85 % at the highest concentration, while DEHP reduced cell viability by >60 % and >40 % at $1x10^{-3}$ M and $1x10^{-4}$ M respectively. Also at $1x10^{-3}$ M, the aldehyde exerted a small but significant reduction while the acid had an insignificant, but noticeable effect. *** P<0.001, ** P<0.01, * P<0.05, Bonferonni post test.

3.2 Effects on Steroidogenesis

The ability of DEHP and its metabolites to disrupt steroidogenesis in MA-10 cells was characterized by acute progesterone production in response to hCG stimulation. Progesterone production was evaluated at 24 hours with concentrations varying from $1x10^{-7}$ M to $1x10^{-4}$ M. These concentrations were chosen because they did not significantly affect cell viability (Figure 3). Data were normalized to total protein to account for differences in cell number from sample to sample.

The results of six independent experiments were analyzed by two-way repeated measures ANOVA. The production of progesterone was dependent on the compound (P=0.0209, two-way ANOVA) and the concentration (P<0.0001, two-way ANOVA), however, as interaction was also significant (P=0.007, two-way ANOVA), one-way repeated measures ANOVA was used to investigate the effect of the concentration for each compound. As shown in Figure 5, DEHP significantly reduced the progesterone production compared to the control at $1x10^{-4}$ and $1x10^{-5}$ M (P<0.001; P<0.05 respectively). DEHP at $1x10^{-}$

^{4} M also reduced the progesterone levels compared to DEHP at $1x10^{-6}$ and $1x10^{-7}$ M ($P<0.01$; $P<0.001$ respectively). MEHP only reduced progesterone significantly compared to the control at the highest concentration, $1x10^{-4}$ M. Finally, the aldehyde reduced the progesterone production compared to the control as well as the other concentrations of DEHP, $1x10^{-5}$, $1x10^{-6}$, $1x10^{-7}$ M ($P<0.001$; $P<0.05$; $P<0.001$; $P<0.001$ respectively). Interestingly, the strongest reduction in progesterone production at the highest concentration of compound was >60 % by 2-ethylhexanal. The second greatest reduction was exhibited by DEHP with a reduction of nearly 45 %. Finally, MEHP reduced progesterone production by over 30 % in MA-10 cells after 24 hours exposure.

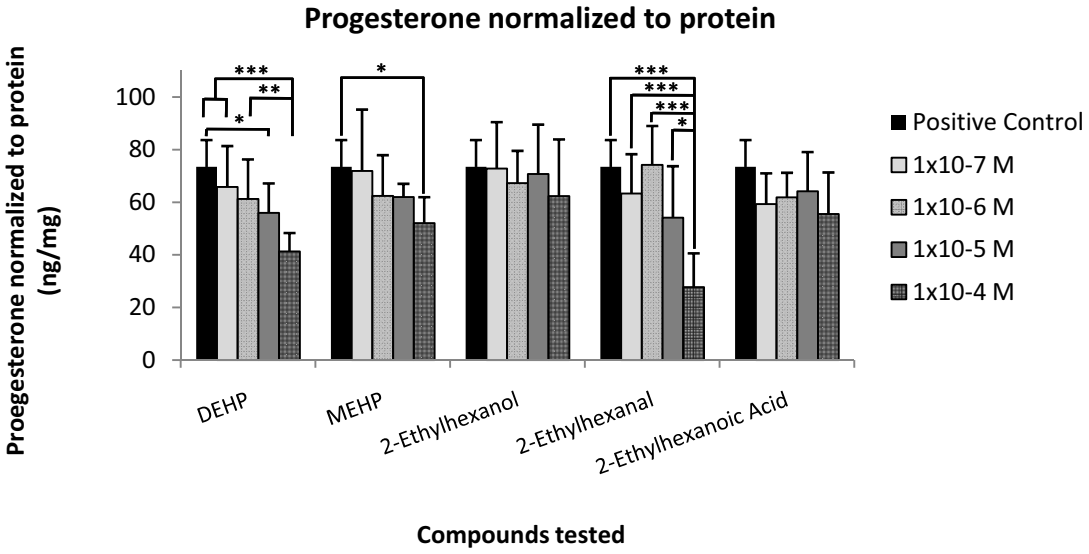


Figure 5 : Progesterone production normalized to total protein in MA-10 cells exposed to DEHP and its metabolites at the concentrations shown for 24 hours, followed by stimulation by 0.5 nM hCG for 4 hours. 2-ethylhexanal reduced progesterone by >60 %, followed by DEHP with a reduction of nearly 45 % and MEHP with a reduction >30 %. Asterix indicate significant difference from control; *** P<0.001, **P<0.01, * P<0.05, Bonferonni post test.

3.3 Gene Expression Analysis

The expression levels of three target genes involved in steroidogenesis were investigated. MA-10 cells were exposed for 24 hours to DEHP or its metabolites at concentrations of $1x10^{-6}$ M, $1x10^{-5}$ M and $1x10^{-4}$ M, followed by maximal stimulation with 0.5 nM hCG for 4 hours. The genes of interest were those encoding the steroidogenic acute regulatory protein (*Star*), the outer-mitochondrial translocator protein (*Tspo*) and cytochrome P450 side chain

cleavage (*Cyp11a1*). Expression was compared to the endogenous reference control gene, *18S*.

The mRNA from 6 independent experiments was quantified by qRT-PCR (relative quantification method) and compared by two-way repeated measures ANOVA, followed by one-way repeated measures ANOVA for each compound.

Two-way ANOVA of *Star* data showed that both the compound and the concentration had significant effects, however, interaction was also significant (P=0.0267, P=0.0002, P=0.0105 respectively). One-way repeated measures ANOVA showed that only MEHP significantly reduced *Star* expression compared to the control (P<0.05) as shown in Figure 6. However non-significant dose-dependent trends may be observed for DEHP and 2-ethylhexanal.

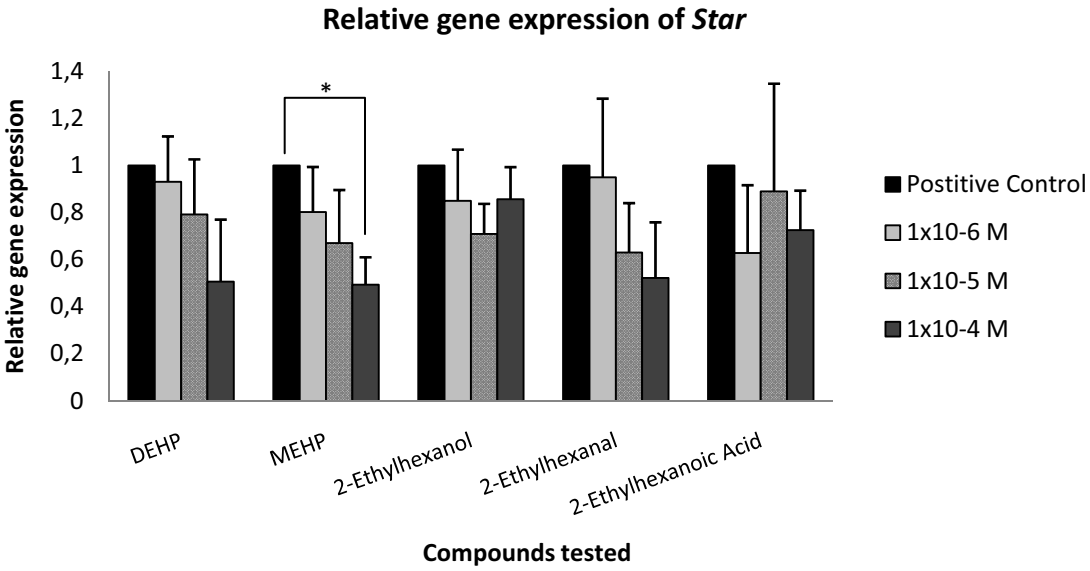


Figure 6 : Relative gene expression of *Star* in MA-10 cells exposed to DEHP and its metabolites at various concentrations and stimulated with hCG. MEHP by 55% at $1x10^{-4}$ M. Asterix indicate significant difference from control; * P<0.05, Bonferonni post test.

The relative gene expression of *Tspo*, a gene that encodes for a protein that works in conjunction with StAR to transport cholesterol across the mitochondrial membrane, was also investigated, as shown in Figure 7. Two-way ANOVA of the data showed that both the compound and the concentration

were significant; however, interaction was significant as well (P=0.0315, P=0.0249, P=0.0174 respectively). One-way repeated measures ANOVA showed that DEHP at $1x10^{-4}$ M reduced the expression of *Tspo* compared to the control (nearly 40 %), as well as DEHP at $1x10^{-5}$ and $1x10^{-6}$ M (P<0.001, P<0.01; P<0.05 respectively). MEHP at $1x10^{-4}$ M also significantly reduced *Tspo* expression, but only compared to MEHP at $1x10^{-6}$ M (P<0.05). Finally, 2-ethylhexanal at the highest concentration reduced expression compared to the control (30 %), and 2-ethylhexanal at $1x10^{-5}$ and $1x10^{-6}$ M (P<0.01; P<0.01; P<0.05 respectively).

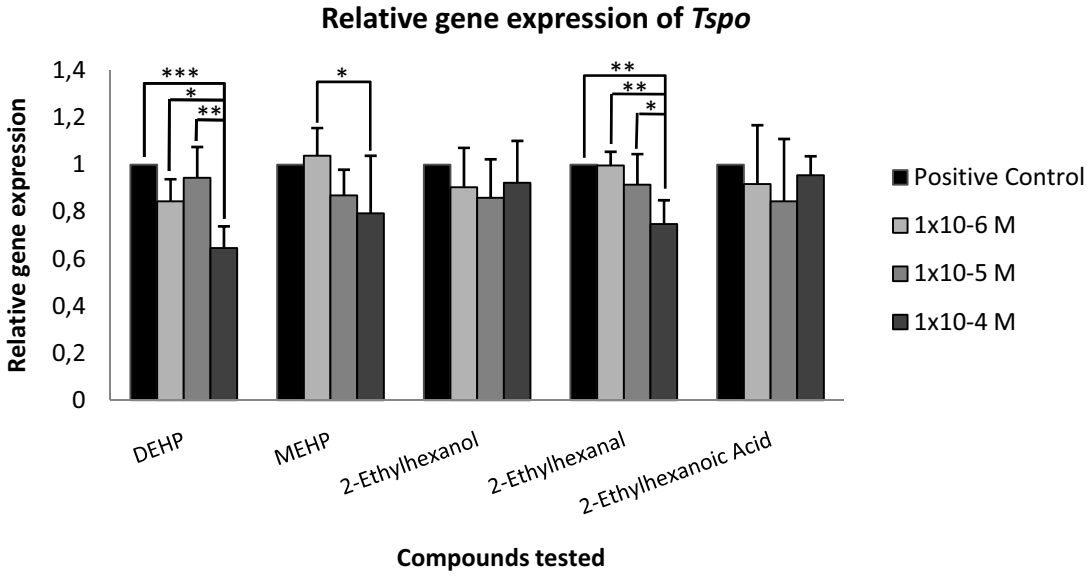


Figure 7 : Relative gene expression of *Tspo* in MA-10 cells exposed to DEHP and its metabolites at various concentrations and stimulated with hCG. DEHP reduced expression compared to the control by 40 % at $1x10^{-4}$ M, MEHP reduced expression by 20% at $1x10^{-4}$ M and 2-ethylhexanal reduced expression by 30% at $1x10^{-4}$ M. Asterix indicate significant difference from control; ***P<0.001, ** P<0.01, *P<0.05, Bonferonni post test.

Finally, the gene expression of *Cyp11a1*, encoding the enzyme responsible for the first enzymatic step in steroidogenesis, conversion of cholesterol to pregnenolone, was explored (Figure 8). Two-way repeated measures ANOVA showed that concentration had a significant effect, as well as interaction (P=0.0035, P<0.0001). One-way repeated measures ANOVA was therefore used to test the effect of concentration for each compound. Only DEHP exerted significant effects, with a reduction in expression by the highest concentration

compared to the control (60%) as well as DEHP at $1x10^{-5}$ and $1x10^{-6}$ M ($P<0.01$; $P<0.001$; $P<0.01$ respectively). Though not significant, a dose-dependent reduction in expression can be observed for MEHP and 2-ethylhexanal.

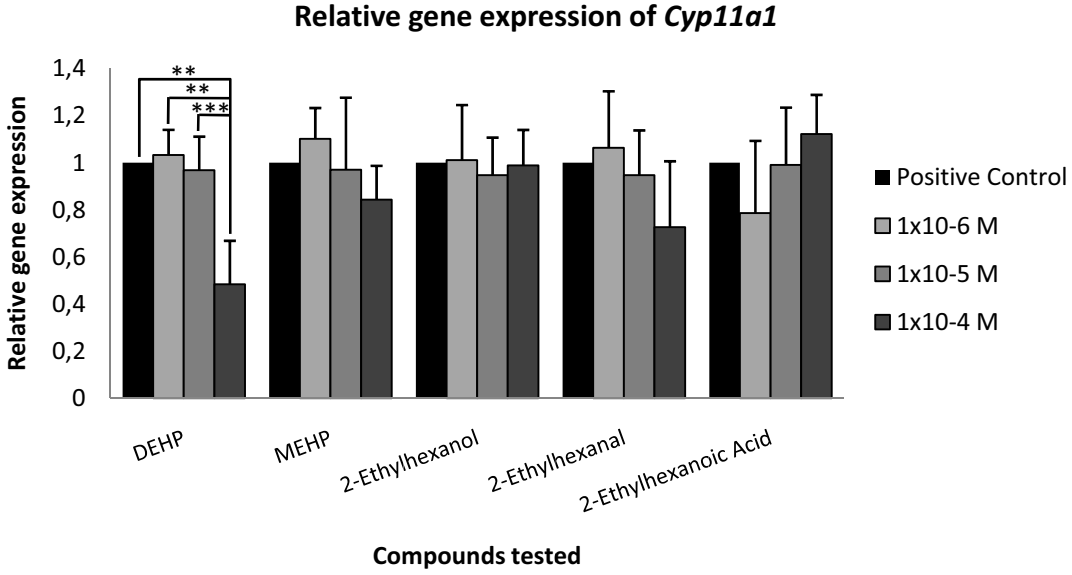


Figure 8 : Relative gene expression of *Cyp11a1* in MA-10 cells exposed to DEHP and its metabolites at various concentrations and stimulated with hCG. DEHP significantly reduced expression by 60 % at $1x10^{-4}$ M. Non-significant dose-dependent trends are observable for MEHP and 2-ethylhexanal. Asterix indicate significant difference from control; *** P<0.001, ** P<0.01, *P<0.05, Bonferonni post test.

4 Discussion

For over sixty years, researchers have been investigating the ability of phthalate plasticizers to induce testicular injury, and the search for the molecular mechanism of action continues today. It has been assumed that the monoester metabolite of DEHP, MEHP, is the active compound responsible for inducing injury[16], though little attention has been given to other metabolites of this plasticizer. The current study investigated the ability of several other metabolites, 2-ethylhexanol, 2-ethylhexanal and 2-ethylhexanoic acid, as well as DEHP and MEHP to affect MA-10 mouse Leydig tumor cells. Cell viability experiments, effects on steroidogenesis and gene expression analysis have shown that MEHP is not the only active metabolite of DEHP; 2-ethylhexanal is capable of disrupting MA-10 cell function as well. In addition, MA-10 cells have been shown to be highly sensitive to the parent compound, DEHP, not just the metabolites.

4.1 Cell Viability

Cell viability experiments showed that after 24 hours exposure of MA-10 cells to each of the compounds, only MEHP significantly reduced cell viability, and only at the highest concentration (Figure 3). However, after 48 hours however (Figure 4), DEHP began to induce a significant decrease in cell viability at the two highest concentrations, though MEHP remained the most potent compound at the highest concentration tested. It is unclear whether this effect of DEHP on MA-10 viability at 48 hours is due to the action of the compound itself, or whether the MA-10 cells have metabolized DEHP to MEHP or another metabolite within this 48 hour time span, and thus the reduction in cell viability is in fact the result of the metabolite(s). No studies investigating the metabolism of DEHP in MA-10 cells have been found, so it cannot be said with certainty that DEHP is not metabolized in 48 hours, however, preliminary studies in HepG2 cells showed that DEHP was only

metabolized by approximately 10 % after 48 hours in these cells[78]. Therefore, if DEHP is only minimally degraded, it may be that MA-10 cells are more sensitive to the parent compound than animal models, or primary cells lines, and may thus represent a conservative model for preliminary high-throughput and rigorous screening of alternative plasticizers. In addition to the effect caused by DEHP and MEHP, a small but significant reduction in cell viability was caused by 2-ethylhexanal, a metabolite that has heretofore been virtually ignored. The alcohol, 2-ethylhexanol, did not have a significant effect on cell viability, nor did the acid, 2-ethylhexanoic acid. However, it should be noted that despite a lack of statistical significance, the highest concentration of the acid did appear to reduce cell viability.

4.2 Effects on Steroidogenesis

The results of the cell viability experiments were used to choose the compound concentrations and exposure time to investigate the ability of DEHP and its metabolites to affect steroidogenesis. This ensured that any effects observed were the result of the compounds acting on the steroidogenic machinery of the cells, not the result of unhealthy cells. Therefore an exposure time of 24 hours and concentrations ranging from $1x10^{-7}$-$1x10^{-4}$ M were chosen as none of the compounds had an effect on cell viability under these conditions. Moreover, since the MTT assay is based on the transformation of MTT to purple formazan crystals by mitochondrial enzymes[91], mitochondrial integrity was not affected under these conditions, an important consideration given that the first steps of steroidogenesis are located in the mitochondria.

Progesterone was used as a marker for steroidogenesis, as MA-10 cells are not capable of producing testosterone. Interestingly, the strongest decrease in progesterone production compared to control was induced by the aldehyde, 2-ethylhexanal, and not MEHP. In fact, even DEHP reduced progesterone to a greater extent than MEHP. These results indicate that while MEHP is in fact an active metabolite, it is not the only metabolite responsible for disrupting steroidogenesis. Whether 2-ethylhexanal might play an important role *in vivo*

is not yet clear, as its distribution within the body following DEHP exposure is not well studied. It is certain, however, that 2-ethylhexanal is capable of disrupting steroidogenesis and should not be overlooked.

Since the current consensus is that MEHP is the active metabolite of DEHP, *in vitro* experiments have focused on the monoester and not DEHP[17,92]. The findings that DEHP has a similar potency with respect to reducing progesterone production in MA-10 cells compared to MEHP parallels the cell viability results and further support the use of MA-10 cells as a screening tool for alternate plasticizers.

4.3 Gene Expression Analysis

Previous studies have shown that phthalates inhibit the gene expression of several key enzymes involved in steroidogenesis, including StAR protein[19,93], TSPO[17] and cytochrome P450scc[19,93]. We investigated whether DEHP and its metabolites could induce similar effects in MA-10 cells. All five compounds were tested at the concentrations used in the progesterone production experiments, excluding the lowest concentration of $1x10^{-7}$ M, as no effects were seen at this concentration, or even at $1x10^{-6}$ M.

The sensitivity of MA-10 cells to the parent compound seen in previous experiments performed in this study was also observed at the gene expression level. All three genes investigated showed decreased expression following exposure to DEHP. As for the aldehyde, expression *Tspo* was significantly reduced, and though not significant for *Star* and *Cyp11a1*, an apparent reduction in expression can be seen. This agrees with the reduced progesterone production caused by 2-ethylhexanal exposure in MA-10 cells. Finally, MEHP also caused reduced expression of the genes, although the results were not significant for *Cyp11a1*. The interesting result that MEHP was not the most potent steroidogenic inhibitor shown in the previous progesterone production experiment is mimicked here in the gene expression of *Tspo*, lending weight to the results.

Curious effects on gene expression as caused by 2-ethylhexanoic acid can be observed from these experiments. While no significant effects were seen for the genes studied, expression was reduced by the lowest concentration tested. The cause of this trend has yet to be determined.

4.4 Agreement with Similar Studies

To the knowledge of this author, there are only two other studies that examined the effects of MEHP in MA-10 cells[17,92]. No other metabolite, or the parent compound, has been tested in this cell line. In 2001, Dees *et al*.[92] were the first to investigate the effects of phthalate esters, in particular MEHP, in the well established Leydig cell model, MA-10 cells. The group reported the ability of the monoester to decrease cell viability and progesterone production at certain concentrations. Some discrepancies are apparent between this group's results and those of the current study, though the overall picture is the same. These discrepancies are namely the concentrations at which effects are seen. In the case of Dees *et al*., progesterone production is affected at $1x10^{-5}$ M, rather than $1x10^{-4}$ M, and cell viability is decreased at $3x10^{-3}$ M and higher, rather than $1x10^{-3}$ M. These minor differences may be caused by experimental differences, such as difference in MA-10 passage number or differences in preparing/measuring the concentration of the MEHP solutions used. The group went on to describe the morphological changes observed in MA-10 cells following exposure to MEHP. They observed an increased number of lipid droplets, and suggested that this effect may be due to the reduced use of cholesterol for steroidogenesis, and proposed that MEHP may interfere at the level of cholesterol transport. The group also noted some morphologically abnormal mitochondria and suggested that mitochondrial damage may be one of the causes of reduced steroidogenesis in MEHP treated MA-10 cells, despite the lack of effects on MTT metabolism.

Other experiments performed by Gazouli *et al*. in 2002[17] on MA-10 cells showed a reduction in progesterone production by MEHP at $1x10^{-5}$ M by approximately 60 %. The results of the current study showed a reduction by

approximately 30 % by MEHP at $1x10^{-4}$ M. The two experiments cannot be compared exactly since Gazouli *et al*. used double the exposure time, 48 hours. Taking this into consideration, the results do agree, as it is likely that with increased exposure time, lower concentrations may begin to exert effects. This group also showed that a similar reduction in testosterone occurred in primary adult rat Leydig cells exposed to MEHP, supporting the use of MA-10 cells as models for disruption of steroidogenesis. The group went on to investigate whether MEHP inhibited mitochondrial transport of cholesterol and the first enzymatic step, conversion of cholesterol to pregnenolone by the P450scc enzyme. They found that MEHP ($1x10^{-4}$ M) inhibited cholesterol transport, which agrees with the gene expression analysis results obtained herein (inhibition of *Star*), as well as the first enzymatic step. Though inhibition of *cyp11A1* (encoding P450scc) was not significant in this study, a trend appeared to be emerging, and it is possible that the effect would be significant after 48 hours exposure. Finally, the group showed that TSPO mRNA was reduced by $1x10^{-4}$ M MEHP by Northern blot analysis, which was also seen with the gene expression analysis of *Tspo* in this study.

4.5 Mechanism of Action

The experiments presented herein show that three of the five compounds tested significantly disrupt steroidogenesis in MA-10 cells. Whether the effect of DEHP is actually due to the rapid metabolism to MEHP or other metabolites in MA-10 cells or not is unclear and metabolic studies of DEHP in this cell line are required in order to clarify this uncertainty. However, previous *in vitro* work with HepG2 cells showed little degradation after 24 hours[78]. It is conceivable that all three of these compounds could exist in a system simultaneously if DEHP is not completely hydrolysed into MEHP and 2-ethylhexanol, and if 2-ethylhexanol is at least partially oxidized to 2-ethylhexanal. If this is the case, all three may be exerting their effects in concert with each other or independently. It is possible that the effects of DEHP *in vivo* are in fact the result of several endocrine disruptors exerting their

effects via different modes of action. In fact, there is already evidence that the anti-androgenic effects of phthalates may be due to more than one mode of action[17,94,95]. Gazouli *et al*. showed in 2002 that the inhibition of testosterone production in mice was partially mediated by PPARα-dependent reduction in TSPO expression, but not entirely as PPARα-null mice were still adversely affected by DEHP[17]. In addition, the multitude of phthalate induced effects in the male reproductive tract supports the idea that multiple modes of action may be at play. For example, it has recently been suggested that the transforming growth factor-β1 (TGF-β1) gene is affected by DEHP and may be involved in the formation of genital tubercules, which may explain the occurrence of hypospadias in rats exposed to DEHP in utero[94]. Testis descent on the other hand is known to be regulated by insulin-like 3 (INSL3), the transcription of which is inhibited by DEHP exposure[95].

There are thus four possibilities; (1) there is only one molecular target with which one key compound (parent or metabolite) interacts (2) there is only one molecular target with which multiple compounds (parent and/or metabolites) interact, (3) there are multiple molecular targets with which one key compound (parent or metabolite) interacts, or (4) there are multiple molecular targets and multiple compounds (parent or metabolite) that interact with one or more of these targets. This study supports the second and fourth possibilities since three of the five compounds inhibited steroidogenesis and suppressed steroidogenic gene expression, and the fourth seems the most likely as there appears to be more than one mode of action of phthalates.

A number of other studies have shown reduced testosterone production by DEHP or MEHP in primary Leydig cells[88] and in Leydig cells *ex vivo*[15,18,88], as well as reduced testicular or circulating testosterone *in vivo*[15,18,88]. Therefore it is well established that DEHP or its active metabolite(s) disrupt the production of testosterone and that such disturbances are likely the cause of the male reproductive abnormalities observed in rodents[96]. In the on-going search for the mechanism of action of phthalates, certain possibilities have been ruled out, such as the binding of DEHP or MEHP to the androgen receptor[56], or the

estrogenic activity of the compounds[97]. A number of other mechanisms have been suggested by various other groups.

One popular suggestion is that DEHP or its active metabolite(s) is a peroxisome proliferator (PP) and is activating the peroxisome proliferator activated receptor (PPAR). This receptor is a member of the nuclear receptor superfamily and acts as a transcription factor in concert with retinoid X receptor (RXR), by binding to the peroxisome proliferator response elements on promoter regions of DNA to regulate the transcription of certain genes. It has been shown that MA-10 cells express certain isoforms of these proteins, namely PPARα and PPARβ/δ[17], and that Leydig cells express RXRα[98], demonstrating that these cells contain the machinery required to interact with PPs. Gazouli *et al*. showed that the impact of DEHP on TSPO mRNA *in vivo* was not seen in PPARα-null mice[17]; however it has also been shown that eliminating PPARα does not eliminate the full effect of DEHP on the testes[99]. It is therefore supposed that while PPAR may play a role in the mediation of DEHP-induced toxicity, it does not act alone; there are PPAR-independent pathways.

One group investigating the short-term and long-term effects of in utero exposure to DEHP in rats showed that some effects differed in fetal (Gestational Day 19 and 20; GD19, GD20), neonatal (Postnatal Day 3; PND3) and adult testes (PND60)[100]. While organ culture of fetal testes showed decreased basal testosterone levels, hCG stimulated testosterone production was not different from controls. In adult animals, circulating testosterone was decreased despite Leydig cell hyperplasia and increased absolute volume of Leydig cells. In addition *Cyp11a1* mRNA levels were decreased in fetal testes, while mRNA levels of *Star* and *Tspo* remained similar to control. In adult testes, the mRNA levels of these genes actually increased, in contrast to the decreased testosterone levels. The increased mRNA levels may be the result of increased Leydig cell number, though this has yet to be resolved. The group also looked at two genes that are considered markers of mature Leydig cells; *Ace2*, which is an adult Leydig cell marker, and insulin-like 3 (*Insl3*), a marker of fully differentiated fetal and adult Leydig cells. While the mRNA levels of

Ace2 were significantly increased in response to DEHP exposure at GD19 and PND3, this was not the case at PND60. As for *Insl3*, expression was decreased in fetal testes but increased in adult testes. The group suggests that the lack of increase in *Ace2* expression in adult cells (despite increased Leydig cell number) and the decrease in fetal *Insl3* expression may indicate a delayed or disrupted maturation process of fetal and adult Leydig cells. In fact, there may be more than one cellular target of in utero DEHP exposure; fetal Leydig cells and/or stem cells of adult Leydig cells. This would explain the differences in effects at different ages and the persistence of effects into adulthood following in utero exposure. Thus evidence of multiple molecular targets is once again presented.

Alterations in the expression of genes in cell survival pathways have been identified by microarray analysis of phthalate exposed rodents[14]. Two such genes are TRPM-2, which is shown to inhibit apoptosis, and bcl-2, which is also known to enhance cell survival in the testes. These genes may account for the Leydig cell hyperplasia associated with fetal DEHP exposure[14].

It has recently been suggested that the effects of DEHP on testes may be the result of oxidative stress[88]. Several groups have detected elevated levels of free radicals in the testes, despite the reduced levels of steroidogenesis (a process which naturally produces free radicals[93]). Kasahara *et al*.[101] and Gautam *et al*.[102] noted that DEHP exposure elevated the levels of reactive oxygen species O_2^- and H_2O_2 in rat testes, while Miura *et al*. noted that high levels of nitric oxides were present in mouse testes[103]. This group also showed that antioxidants such as glutathione are reduced in DEHP treated mouse testes, while application of antioxidants such as vitamin C and E can mitigate the effects of DEHP on spermatogenesis[103]. While these groups focused on the effects of DEHP on germ cells, oxidative stress has been linked to suppression of StAR expression in rat Leydig cells[104], suggesting that oxidative stress could also play a role in deleterious effects of DEHP on Leydig cells.

In March 2009, Lee *et al*. investigated the effect of chronic DEHP exposure in adult male rats on the regulation of steroidogenesis and spermatogenesis[89].

In addition to examining the expression of the genes involved in steroidogenesis, the group also looked at the expression of thyroid receptor alpha 1 (TRα1), as hormone receptor cross talk may be important in testes development. They found that TRα1 expression was significantly increased in DEHP treated rats, and propose that this receptor may play an important role in DEHP-induced testicular dysgenesis[89]. In addition, the expression of luteinizing hormone receptor (LHR) has been shown to be inhibited by DEHP[93]. This may play a role in the ability of late fetal Leydig cells to respond to hormone stimulation, though this is not suspected to be the case in early fetal cells as they do not yet express the LH receptor[105].

Other possible modes of action for testosterone suppression that have been suggested include altered gene expression by interaction of DEHP or its active metabolite(s) with the aryl hydrocarbon receptor (a transcription factor most well known for its binding to dioxins), or disrupted communication between Leydig and Sertoli cells[88].

4.6 2-Ethylhexanal

In an attempt to understand why 2-ethylhexanal might exert such strong effects on Leydig cell steroidogenesis, a search for other aldehydes known to cause similar effects in the testes has been performed. Review of the literature linked the consumption of alcohol and its metabolism to testicular injury. The fact that alcohol consumption can cause testicular injury in animals as well as humans has long been known[106-108]. In 1983, Van Thiel *et al*. compared ethanol exposure *in vivo* and *in vitro* and found that testosterone secretion was reduced, and testicular damage (reduced testes and seminiferous tubules, fewer or no germ cells) was observed *in vivo*. Furthermore, administration of physiologically relevant concentrations of acetaldehyde, a metabolite of ethanol resulting from the oxidation of ethanol by the enzyme alcohol dehydrogenase, reduced testosterone production *in vitro*. Cicero *et al*.[109,110] reported similar findings in testicular cell preparations treated with ethanol and acetaldehyde. In

fact, this group found that acetaldehyde was up to 4000 times more potent than ethanol[110].

One of the proposed modes of action for testicular injury caused by ethanol is oxidative stress[111-113]. The presence of lipid peroxidation, lower antioxidant (ex. glutathione) levels[111], free radicals and abnormal mitochondria in Leydig cells following alcohol treatment support this hypothesis[114]. Most studies have focused on the oxidation of ethanol to acetaldehyde, but the effects of acetaldehyde on Leydig cells indicate that its oxidation to acetic acid by the aldehyde dehydrogenase enzyme may be important as well, as the presence of this enzyme in Leydig cells has been confirmed[115].

Drawing a parallel between acetaldehyde and 2-ethylhexanal, the effect of the aldehyde in this study may therefore be due to its oxidation to 2-ethylhexanoic acid and the resulting production of free radicals. But if this is possible, shouldn't the oxidation of 2-ethylhexanol to 2-ethylhexanal also exert an effect? The lack of effect observed in this study may be attributable to the fact that the aldehyde may simply be much more potent than the alcohol, as in the case of acetaldehyde and ethanol[109]. It is also possible that alcohol dehydrogenase is less active than aldehyde dehydrogenase in MA-10 cells. Metabolic studies in MA-10 cells are required to test this hypothesis, as well as dose-response studies covering a broader range of concentrations of 2-ethylhexanol and 2-ethylhexanal. Thus the mode of action of 2-ethylhexanal may be oxidative stress, as has previously been suggested as one of the mechanisms of toxicity of DEHP.

4.7 Relevance of Concentrations Used

While the general population is exposed to relatively low levels of DEHP, there are certain subsets of the population that are exposed to much higher levels. For example, neonates requiring intensive care are exposed to high concentrations of phthalates from the use of plastic medical equipment, such as IV tubing, umbilical catheters, blood bags, etc. Some groups have reported urinary concentrations of MEHP in neonatal intensive care units on the order of

$3x10^{-6}$ M[116,117]. This concentration is deceptive, however, since MEHP is a minor urinary metabolite, and thus exposure levels would in fact be much higher[118]. The concentrations of other metabolites, such as 5OH-MEHP, were on the order of $1x10^{-3}$ M[76,116]. Therefore the concentrations used in this study, ranging from $1x10^{-7}$-$1x10^{-3}$ M, are quite relevant to human health. It is also important to note that outside the laboratory, humans are exposed to a cocktail of phthalates and other possible endocrine disruptors that may behave in an additive or synergistic manner. Taking this into consideration, it is conceivable that the rising occurrence of testicular dysgenesis syndrome among humans[8] is due, at least in part, to phthalate exposure.

4.8 Future Work

Research investigating the mechanism(s) of action of phthalates in testicular injury has intensified over the last decade, and it is clear that while more research is needed, it is only a matter of time before the mystery is unveiled. This will also shed light on novel regulatory mechanisms involved in steroidogenesis, and help to understand how non-traditional endocrine disruptors work. In addition, this knowledge will aid in the development of new and safe plasticizers that will not interfere with male reproductive tract development.

In addition to mechanism based research, investigating the remaining metabolites of DEHP and other plasticizers could reveal other potentially hazardous compounds, as did this study. Only one study to date performed by Chauvigne *et al*. investigates the effects of some of the oxidized metabolites of MEHP[119]. This group recently tested the effects of DEHP, MEHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (5OXO-MEHP) and mono-(2-ethyl-5-carboxy-pentyl) phthalate (5CX-MEPP) on an organoculture of fetal rat testes. They showed that in addition to MEHP, 5OH-MEHP significantly decreased gonocyte number and basal testosterone production[119]. Taking the results of the current study, and

that performed by Chauvigne *et al*., it is evident that while MEHP does induce testicular injury, there appears to be more than one active metabolite.

As mentioned earlier, the ability of MA-10 cells, as well as other Leydig cells, to metabolize DEHP has not been tested. Such studies would help to resolve whether the compounds administered to *in vitro* cultures remain in their original form, or if they are transformed into metabolites that exert deleterious effects on host cells. In addition, the disposition of DEHP and all of its metabolites within the body following various routes of administration would help to define the risks associated with DEHP exposure.

5 Conclusion

The results of the study described herein demonstrated that DEHP and two of its metabolites, MEHP and 2-ethylhexanal, are capable of not only interfering with cell viability in MA-10 cells, but also disrupting steroidogenesis at the gene expression and hormone levels. This implies that the hypothesis that MEHP is the only active metabolite of DEHP was premature, and that 2-ethylhexanal and possibly other metabolites not yet studied may contribute to the testicular toxicity of DEHP. The finding that more than one metabolite is active supports the growing evidence for numerous mechanisms of action, and several molecular targets in the induction of testicular injury. In fact, the activity of several compounds may help to explain this plurality of possible mechanisms. Paralleling the effects of 2-ethylhexanal to acetaldehyde and its potential to produce testicular injury suggested one possible mode of action for this particular metabolite: oxidative stress. There is also evidence of free radical damage in animal models exposed to the parent compound, supporting this hypothesis. Finally, this study demonstrates that MA-10 cells are an appropriate cell line for the high-throughput screening of current and future plasticizers for anti-androgenic effects and the results presented will provide a suitable *in vitro* basis for comparison of new plasticizers.

6 References

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Appendix I: Chemicals and Suppliers

Product Name/Description	Supplier	Catalogue/Model #
-80 ^{0}C freezer	Thermo Electron Corporation	Forma -86C ULT freezer
-20 ^{0}C freezer	Fisher Scientific	Isotemp
2-ethylhexanal	Sigma-Aldrich	E29109
2-ethylhexanoic acid	Sigma-Aldrich	538701
2-ethylhexanol	Sigma-Aldrich	538051
20 $^{1}/_{2}$ gauge needle, blunt	BD (Fisher)	305175
2N Hydrochloric acid (HCl)	Fisher	SA431
Adjustable Volume Research® Pipettes	Eppendorf	0.5-10 µL, 022471902 20-200 µL, 022472054 100-1000 µL, 022472101
Anhydrous ethyl alcohol	Commercial Alcohols	8507
Autoclave	STERIS®	Amsco® Lab 250 Small Steam Sterilizer
β-mercaptoethanol	Sigma-Aldrich	M-7154
Bibulous (absorbent) paper	Fisherbrand (Fisher)	11-998
Bicinchoninic acid (BCA) Protein Assay Kit	Thermo Scientific	23227
Biosafety cabinet (BSC)	Thermo Electron Corporation	Forma Class II, A2 BSC
Cell culture plates	Corning Costar® (Fisher)	96-well-3596 6-well-3506
Centrifuge tubes, 15 mL	Cellstar (Fisher)	188 261
Centrifuge tubes, 50 mL	Fisher	0334114A
Centrifuge	Thermo Scientific	IEC Centra CL2
Chorionic Gonadotropin, Human	Calbiochem	869031

Urine, Iodination Grade (≥12 000 IU/mg)		
Corning T-75 flasks with vented caps	Corning (Fisher)	430641
Coulter counter	Beckman Coulter	Z2 Coulter® Particle Count and Size Analyzer
Cryogenic Vials (2 mL)	Corning (Fisher)	430659
Di(2-ethylhexyl) phthalate	Sigma-Aldrich	80030
Dimethyl Sulfoxide (DMSO)	Fisher	BP231
Filter pipette tips	STARSTEDT (Fisher)	2.5 µL-70.1130.212 10 µL-70.1130.210 20 µL-70.760.213
	Molecular Bioproducts (Fisher)	200 µL-2069 1000 µL-2079E
Filters, 0.22 µm	Millipore (Fisher)	SLGV M33 RS
Gelatin from porcine skin, Type A	Sigma-Aldrich	G2500
Hemacytometer	Hausser (Thomas Scientific)	5971R10
Horse serum (heat inactivated)	Gibco (Invitrogen)	26050
HEPES buffer (1M)	Gibco (Invitrogen)	15630
High Capacity cDNA Reverse Transcription kti	ABI	4368814
Incubator	Thermo Electron Corporation	Model Forma Series II Incubator
Life Science Analyser (UV/Vis spectrophotometer)	Jenway (Barloworld Scientific)	Genova MK3
Minimum Essential Medium (MEM) Alpha 1X (phenol red free)	Gibco (Invitrogen)	41061
MicroAmp® 384-well clear optical reaction plate	ABI	4309849
MicroAmp® Fast Optical 96 well reaction plate	ABI	4346906

MicroAmp™ Optical Adhesive Film kit	ABI	4313663
Microcentrifuge	Thermo Scientific	Micromax RF
Microplate spectrophotometer	Bio-Rad Laboratories, Inc	Benchmark Plus
Microscope	Leica	Leica DM IL
Mono(2-ethylhexyl) phthalate	Crescent Chemical Co., Inc	C161789
MTT	Sigma-Aldrich	M5655
Multiply® Pro cup PCR tubes, 0.2 mL	STARSTEDT (Fisher)	72.737.002
Nuclease-free water	Qiagen	129114
Penicillin-Streptomycin	Gibco (Invitrogen)	15140
Phosphate Buffered Saline 10X (without Calcium and magnesium)	Multicell (Wisent, Inc)	311-012-CL
Pipette tips	Eppendorf (Fisher)	10 µL - 21-197-2F 200 µL - 02-707-121 1 mL - 02-707-123
Power SYBR® Green Master Mix	ABI	4367659
Primers	Alpha DNA	Sequence specific
Progesterone ELISA	Fitzgerald Industries International, Inc.	55R-RE52231
QIAshredder	Qiagen	79654
Quantitative PCR machine	ABI	7900HT Fast Real-Time PCR system
Quartz Micro cuvette	Jenway	035-139
RNase-free DNase set	Qiagen	79254
RNeasy Mini kit	Qiagen	74104
Sodium Chloride	Fisher	S671
Sodium Deoxycholate	Sigma-Aldrich	D6750
Sodium Dodecyl Sulfate (SDS)	Fisher	BP166
Syringes, 1 mL	BD (Fisher)	309602

Tergitol Solution (NP40S) 70% in water	Sigma-Aldrich	NP40S
Thermal Cycler	Bio-Rad	iCycler
Tris Base	Fisher	BP152
Trypsin-EDTA·4Na 1X, 0.05%	Gibco (Invitrogen)	25300
Vortex mixer	Fisher Scientific	02215360
Waymouth's MB 752/1 (1X) medium	Gibco (Invitrogen)	11220

Appendix II: Biohazard Approval

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

Projects involving potentially biohazardous materials should not be commenced without approval from Environmental Health & Safety. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: David Cooper PHONE: x4278

DEPARTMENT: Chemical Engineering FAX: x6678

ADDRESS: 3610 University St E MAIL: david.cooper@mcgill.ca or richard.leask@mcgill.ca

PROJECT TITLE:Green plasticizers

2. EMERGENCY: Person(s) designated to handle emergencies

Name: Richard L. Leask Phone No: work: x4270 home: 514-369-7996

Name: Alain Gagnon Phone No: work: x4490 home: Security x3000

3. FUNDING SOURCE OR AGENCY (specify): NSERC Strategic

Grant No.: TBA Beginning date: Oct 1, 2007 End date: Sept 30, 2010

4. Indicate if this is

☐ Renewal: procedures previously approved without alterations.

Approval End Date: _____

☐ New funding source: project previously reviewed and approved under an application to another agency

Agency: _____ Approval End Date: _____

☒ New project: project not previously reviewed.

☐ Approved project: change in biohazardous materials or procedures.

☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: Environmental Health & Safety approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in Health Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☐ 1 ☒ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: _____ (SIGNATURE) date: 16 (day) 10 (month) 2007 (year)

Approved by Environmental Health & Safety: _____ (SIGNATURE) date: 26 (day) 10 (month) 07 (year)

Expiry: 30 (day) 09 (month) 10 (year)

*as defined in the "McGill Laboratory Biosafety Manual"

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes, indicate training date.
Carlie Piche	Chemical Engineering	MEng	Yes, 2006

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

- The response of mammalian cells (HEPG2, WIFB) and bacterial cells (Rhodococcus sp. and other soil microbes) to common plasticizers (and newly synthesized) will be examined

ii) the procedures involving biohazards

Cell culture (mammalian/bacterial cells), immunostaining, Westernblots., PCR

iii) the protocol for decontaminating spills

As per section 5.3 of the Mcgill biosafety manual.

1.Wear gloves and a laboratory coat or gown. Heavyweight, puncture-resistant utility gloves, such as those used for housecleaning and dishwashing..

2.Do not handle sharps with the hands. Clean up broken glass or other sharp objects with sheets of cardboard or other rigid, disposable material. If a broom and dustpan are used, they must be decontaminated later.

3.Avoid generating aerosols by sweeping.

4.Absorb the spill. Most disinfectants are less effective in the presence of high concentrations of protein, so absorb the bulk of the liquid before applying disinfectants. Use disposable absorbent material such as paper towels. After absorption of the liquid, dispose of all contaminated materials as waste.

5.Clean the spill site of all visible spilled material using an aqueous detergent solution (e.g., any household detergent). Absorb the bulk of the liquid to prevent dilution of the disinfectant.

6.Disinfect the spill site using an appropriate disinfectant, such as a household bleach solution. Flood the spill site or wipe it down with disposable towels soaked in the disinfectant.

7.Absorb the disinfectant or allow it to dry. 8.Rinse the spill site with water.

9.Dispose of all contaminated materials properly. Place them in a biohazard bag or other leakproof, labeled biohazard container for sterilization.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

No

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

No

9. What precautions will be taken to reduce production of infectious droplets and aerosols?

All work will be conducted in a ~~laminar flow hood~~ and universal procotions will be observed.

biological safety cabinet

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.

No

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.

No

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Wong	7050	Thermo	1286	101513-2110	May 2005

Appendix III: Protocols

Cell Culture

Materials

1. Waymouth's MB 752/1 (1X) media (Invitrogen)
2. HEPES (1M) buffer (Invitrogen)
3. Horse serum (Heat inactivated) (Invitrogen)
4. Penicillin-Streptomycin (Invitrogen)
5. Calcium and magnesium free phosphate buffered saline (PBS) (Wisent, Inc.)
6. 0.05% Trypsin-EDTA·4Na 1X (Invitrogen)
7. Pig gelatin (0.1%)
8. Microscope
9. Centrifuge
10. Hemacytometer or Coulter counter
11. T-75 Corning flasks
12. 2 mL Cryogenic vials
13. Water bath
14. Crushed ice

Protocol: Trypsinizing

1. Remove media from flask
2. Add 5 mL trypsin to flask and incubate at room temperature for 35 seconds
3. Remove trypsin and incubate flask at 37 °C and 5% CO2 for 1-2 minutes
4. Add 5-10 mL warm media to flask, detaching cells with shear
5. Pipette up and down several times to break up clumps of cells
6. Count cell suspension with hemocytomer or coulter counter (see appropriate protocols for uses of cell suspensions)
7. Protocol: Expanding
8. Coat three T-75 flasks with pig gelatin for each T-75 flask being split, incubate 40 minutes at 37 °C and 5% CO2
9. Trypsinize as described in the trypsinizing protocol
10. Split cell suspension evenly into three new flasks after removing gelatin and complete media volume to 20 mL for a T-75 flask
11. Identify new flasks with cell line, date, cell concentration and passage number
12. Incubate flasks 37 °C and 5% CO2
13. Change media every second day, split every 3-5 days
14. Protocol: Freezing

15. Prepare cold freezing media; 90% media, 10 v/v % DMSO
16. Trypsinize cells as described in the trypsinizing protocol
17. Centrifuge cell suspension at 500 g for 5-10 minutes to pellet cells
18. Carefully remove media without disrupting cell pellet
19. Resuspend cells from one confluent T-75 in 5-6 mL of cold freezing media
20. Pipette 1 mL of cell suspension into freezing ampoules, label and place in an insulated container (ex: Mr. Frosty) that will allow the cell suspension to cool slowly.
21. Place ampoules in -80 °C freezer overnight, then transfer ampoules to cryotank.
22. Protocol: Thawing
23. Coat oneT-75 flask with pig gelatin for each ampoule being thawed, incubate 40 minutes at 37 °C and 5% CO2
24. Remove gelatin from flask and add 10 mL warm media
25. Remove ampoule from cryotank and place on ice for 1 minute
26. Swirl ampoule in 37 °C water bath to thaw (1-2 min.) and transfer cell suspension to flask with 10 mL media rapidly after wiping down ampoule with 70 % ethanol
27. Swirl flask and incubate at 37 °C and 5% CO2
28. Replace the media with 20 mL fresh media the next day to remove any residual DMSO from the freezing media

Cell Viability

Materials

1. 96-well plate
2. 0.1% pig gelatin
3. Microscope
4. Hemacytometer or coulter counter
5. Bio-Rad laboratories Benchmark Plus microplate spectrophotometer (or equivalent)
6. Cell culturing materials (media, trypsin, etc.) (Invitrogen)
7. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 12 mM stock solution in PBS (Sigma Aldrich)
8. Calcium and magnesium free PBS (Wisent, Inc)
9. DMSO
10. Media with compounds of interest at various concentrations
11. SDS-HCl detergent
12. Minimum Essential Medium (MEM) Alpha 1X (phenol red free)

Protocol

1. Coat 96-well plate with 0.1% pig gelatin and incubate at 37 °C for 40 minutes
2. Trypsinize confluent flask of MA-10 cells and resuspend in 5 mL of media
3. Count cells with hemacytometer or coulter counter
4. Prepare 6 to 8 cell suspensions of different concentrations for a standard curve (ex: 10 000, 20 000, 40 000, 50 000, 60 000, 80 000 and 100 000 cells/mL)
5. Prepare 10 mL of cell suspension at 50 000 cells/mL for sample wells
6. Remove pig gelatin from 96-well plate and add 100 µL of cell suspension to each well (5000 cells/well); this should include triplicates for each standard curve and sample well (see sample plate setup below for details)
7. Include 3 control wells (cell concentration: 50 000 cells/mL) on the plate as well as 3 blank wells (no cells, just media)
8. Incubate cells for 24 hours at 37 °C and 5% CO2
9. Remove media from all wells, add media with compounds at various concentrations to sample wells, add media with 0.3 v/v % DMSO to standard curve, control and blank wells
10. Incubate plate for 24 or 48 hours at 37 °C and 5% CO2
11. Near end of incubation time, prepare 11 mL of MTT media; add 1 mL of MTT stock solution to 10 mL clear media
12. At end of incubation time, remove media from each well, wash once with PBS and add 110 µL of MTT media to each well
13. Incubate plate for 4 hours at 37 °C and 5% CO2 to allow cells to metabolize MTT and form purple crystals
14. Add 100 µL of SDS-HCl detergent to each well to solubilize purple crystals, incubate 18 hours at 37 °C and 5% CO2
15. Read plate with microplate spectrophotometer at 570 nm

Disruption of Steroidogenesis

Materials

1. Ten 6-well plates
2. 0.1% pig gelatin
3. Microscope
4. Hemacytometer or coulter counter
5. Cell culturing materials (media, trypsin, etc.) (Invitrogen)
6. Calcium and magnesium free PBS (Wisent, Inc)
7. DMSO
8. Media with compounds of interest at various concentrations
9. Human Chorionic Gonadotropin (hCG), Potency: 12 500 IU/mg

Protocol

1. Coat ten 6-well plate with 0.1% pig gelatin and incubate at 37 °C for 40 minutes
2. Trypsinize confluent flask of MA-10 cells and resuspend in 5 mL of media
3. Count cells with hemacytometer or coulter counter, and dilute suspension to 25 000 cells/mL
4. Remove pig gelatin from 6-well plates and seed 1.6 mL cell suspension in each well, incubate at 37 °C and 5% CO2 for 24 hours
5. Remove media from all wells, add media with compounds at various concentrations to sample wells, add media with 0.3 v/v % DMSO to control wells. Control wells include positive controls (PC) which are incubated in media with 0.3 v/v % DMSO and stimulated with hCG in step 8, and negative controls (NC) which are incubated in media with 0.3 v/v % DMSO, but not stimulated with hCG.
6. Incubate plate for 24 hours at 37 °C and 5% CO2
7. Remove media and wash once with PBS
8. Stimulate cells with 1.6 mL 0.5 nM human chorionic gonadotropin (hCG) in media (add media only to NC wells), incubate at 37 °C and 5% CO2 for 4 hours
9. Collect media for analysis with progesterone ELISA and store at -20 °C
10. Rinse cells twice with PBS before lysing for total protein measurement

Progesterone Measurement

Based on protocol provided by kit manufacturer

Materials

1. Progesterone Enzyme-Linked Immunoassay (ELISA) kit (Fitzgerald Antibodies, Inc)
    a. 96 microtiter wells coated with anti-Progesterone antibody
    b. Progesterone standards (0, 0.3, 1.25, 2.5, 5, 15, 40 ng/mL)
    c. Enzyme Conjugate (Progesterone conjugated to horseradish peroxidase)
    d. Substrate Solution
    e. Stop Solution
    f. Wash Solution
2. Bio-Rad laboratories Benchmark Plus microplate spectrophotometer (or equivalent)
3. Bibulous (absorbent) paper
4. Distilled/deionized water
5. Micropipettes

Protocol

1. Allow all reagents, samples and materials to reach room temperature before starting
2. Dilute 40X wash buffer (blue cap) to 1X: mix 5 mL of 40X wash buffer with 195 mL deionized water
3. Secure the desired number of Microtiter wells in the holder
4. Add 25 µL of each standard, sample and control in different wells in duplicate or triplicate
5. Incubate for 5 minutes at room temperature
6. Add 200 µL of Enzyme Conjugate into each well (pink solution in bottle with white cap)
7. Mix thoroughly with plate reader for 10 seconds
8. Incubate plate for 60 minutes at room temperature
9. Shake out contents of wells and rinse with 400 µL 1X wash buffer three times, striking the plate sharply on absorbent paper between each wash to remove excess droplets
10. Add 200 µL of Substrate Solution (brown bottle, yellow cap) to each well
11. Incubate 15 minutes at room temperature
12. Add 100 µL Stop Solution (red cap) to each well to stop the enzymatic reaction
13. Read the absorbance at 450 nm with the microplate spectrophotometer within ten minutes of stopping the reaction
14. To calculate the unknown progesterone concentrations, fit a 4PL curve to the standard curve

Total Protein Measurement

Based on protocol provided by kit manufacturer

Materials

1. Bicinchoninic acid (BCA) Protein Assay Kit (Pierce)
    a. BCA Reagent A
    b. BCA Reagent B
    c. Bovine Serum Albumin (BSA) Standard Ampoules (2 mg/mL)
2. Bio-Rad laboratories Benchmark Plus microplate spectrophotometer (or equivalent)
3. Pipettes
4. RIPA lysis buffer

Protocol

1. Prepare cells as described in Steroidogenesis Disruption Experiment protocol
2. Lyse dry cells in 6 well plates by adding 400 µL RIPA buffer to each well
3. Shake plates thoroughly for 10 minutes

4. During lysis, prepare a standard curve by diluting the BSA standard provided with RIPA lysis buffer to 6-8 desired concentrations (ex: 0, 25, 50, 75, 100, 150, 200 µg/mL)
5. Ensure cells are fully lysed visually with light microscope
6. Pipette 25 µL of standard, samples and controls into a 96 well plate in duplicate or triplicate
7. Prepare Working Reagent by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B; prepare sufficient Working reagent for 200 µL/well
8. Add 200 µL Working reagent to each well, mix 1 minute, incubate at 37 ^{0}C for 30-120 minutes (longer incubation times lower the minimum detection level of the reagent as well as working range of the assay, which may be desirable for low protein concentrations)
9. Read the absorbance with a microplate spectrophotometer at 562 nm; use a linear, four-parameter (quadratic) or best-fit curve to calculate the unknown concentrations from the standard curve

Gene Expression Experiment

Materials

1. 6-well plates
2. 0.1% pig gelatin
3. Microscope
4. Hemacytometer or coulter counter
5. Cell culturing materials (media, trypsin, etc.) (Invitrogen)
6. Calcium and magnesium free PBS (Wisent, Inc)
7. DMSO
8. Media with compounds of interest at various concentrations
9. Human Chorionic Gonadotropin (hCG), Potency: 12 500 IU/mg

Protocol

1. Coat ten 6-well plate with 0.1% pig gelatin and incubate at 37 °C for 40 minutes
2. Trypsinize confluent flask of MA-10 cells and resuspend in 5 mL of media
3. Count cells with hemacytometer or coulter counter, and dilute suspension to 200 000 cells/mL
4. Remove pig gelatin from 6-well plates and seed 1.6 mL cell suspension in each well, incubate at 37 °C and 5% CO2 for 24 hours
5. Remove media from all wells, add media with compounds at various concentrations to sample wells, add media with 0.3 v/v % DMSO to control wells. Control wells include positive controls (PC) which are incubated in media with 0.3 v/v % DMSO and stimulated with hCG in step 8, and negative controls (NC) which are incubated in media with 0.3 v/v % DMSO, but not stimulated with hCG.

6. Incubate plate for 24 hours at 37 °C and 5% CO2
7. Remove media and wash once with PBS
8. Stimulate cells with 1.6 mL of 0.5 nM human chorionic gonadotropin (hCG) in media (add media only to NC wells), incubate at 37 °C and 5% CO2 for 4 hours.
9. After incubation period, remove media, rinse twice with PBS and proceed to RNA extraction section below.

RNA Extraction

Protocol based on that of Qiagen RNeasy kits. Special care must be taken when handling RNA as it is easily degraded. Read the handbook supplied if unfamiliar with handling RNA. Do not mix Buffers RLT and RW1 with bleach.

Materials

1. RNeasy Mini kit (Qiagen)
    a. RNeasy mini spin columns (pink)
    b. Collection tubes (1.5 and 2 mL)
    c. Buffer RLT
    d. Buffer RW1
    e. Buffer RPE
    f. RNase-free water
    g. Handbook
2. RNase-free DNase set (Qiagen)
    a. Lyophilized DNase I
    b. Buffer RDD
    c. RNase-free water
3. QIAshredder columns (Qiagen)
4. Microcentrifuge
5. -80^{o}C freezer
6. 20 ½ gauge needle
7. 1 mL syringes
8. RNase-free water
9. 96-100% ethanol
10. RNase-free filter tips and pipettes
11. RNase-free 70% ethanol solution in water
12. β-mercaptoethanol

Protocol

1. Add 10 µl β-mercaptoethanol (β-ME) per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing
2. Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution

3. Prepare DNase I stock solution: Using an RNase-free needle and syringe, inject 550 µl RNase-free water into the DNase I vial (1500 Kunitz units). Mix gently by inverting the vial. Do not vortex. For long-term storage of DNase I stock solution, divide it into single-use aliquots and store at –20 °C for up to 9 months. Thawed aliquots can be stored at 2–8 °C for up to 6 weeks. Do not refreeze aliquots after thawing
4. Harvest cells prepared as described in the "Gene expression experiment" section above by adding 350 µL of Buffer RLT to each well and pipetting to mix
5. Pass the lysate 6 times through a blunt 20 ½ gauge needle fitted to an RNase free syringe to shear DNA
6. Pipette the lysate into a QIAshredder spin column placed in a 2 mL collection tube and centrifuge for 2 minutes at full speed
7. Add 350 µL of 70% ethanol to the homogenized lysate and mix well by pipetting
8. Transfer lysate, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 mL collection tube. Close the lid and centrifuge at 8000 x g for 30 seconds. Discard flow-through.
9. Reusing collection tube from step 8, add 350 µL Buffer RW1 to RNeasy column, close lid and centrifuge for 30 seconds at 8000 x g, discard flow-through.
10. Add 70 µL Buffer RDD to 10 µL aliquots of DNase I stock solution, mix gently by inverting tube (do not vortex, shear may damage DNase enzyme), centrifuge briefly.
11. Add 80 µL DNase I incubation mix directly to RNeasy colum membrane, making sure to cover membrane entirely, and place on benchtop for 15 minutes
12. Add 350 µL Buffer RW1 to RNeasy column, close lid, centrifuge for 30 seconds at 8000 x g, discard flow-through.
13. Add 500 µL Buffer RPE to the RNeasy spin column. Close lid, centrifuge for 30 seconds at 8000 x g to wash the spin column membrane. Discard flow-through.
14. Add 500 µL Buffer RPE to the RNeasy spin column. Close lid, centrifuge for 2 minutes at 8000 x g to wash the spin column membrane. Discard flow-through.
15. Without adding any buffer, place the RNeasy spin column in a new 2 mL collection tube and place in the centrifuge. Close the lid gently, and centrifuge at full speed for 1 min.
16. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 40 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 8000 x g to elute the RNA.
17. Repeat step 16 using the eluate.
18. Store RNA at -80 °C.

RNA quantification

Materials

1. Genova MK3 Life Science Analyser (or similar UV/Vis Spectrophotometer) and cuvette
2. Distilled, deionized water (ddH2O)
3. Pipettes and disposable tips
4. Microcentrifuge tubes
5. Compressed air source

Protocol

1. Turn on Life Science Analyser (if it was recently turned off, wait 20 minutes before turning it back on).
2. Select "DNA/RNA" mode option from the main menu.
3. Select the "260/280nm" mode of operation.
4. Select "SETUP" and enter the dilution factor (0005+0120µL), set the resolution to the highest (0.001) and select "EXIT".
5. Place a blank sample of ddH2O in the sample chamber (be sure to rinse and dry the cuvette several times between readings).
6. Press "CAL" to calibrate the instrument.
7. Dilute sample RNA by mixing 5 µL of RNA in 120 µL ddH2O. Mix well and centrifuge briefly.
8. Remove blank from sample chamber, discard water and dry. Pipette 125 µL sample into cuvette.
9. Place cuvette in sample chamber and select "READ".
10. Record absorbance at λ 260, λ 280 and the ratio (260:280).
11. The concentration of RNA is equal to the abosorbance260 X dilution factor X 40 ng/µL.
12. Good quality RNA should have a 260:280 ratio between 1.8-2.

Name	Value
Factor 1	62.9 (default)
Factor 2	26.0 (default)
Dilution	0.0005+0.120 µL
Correction	NO
Wavelength	320 (default)
Units	µg/mL
Resolution	0.001

Primer Design

In order to amplify cDNA, it is necessary to design a primer for the desired gene. One can either use primers found in literature, order guaranteed primers from suppliers, or design his/her own using the following procedure.

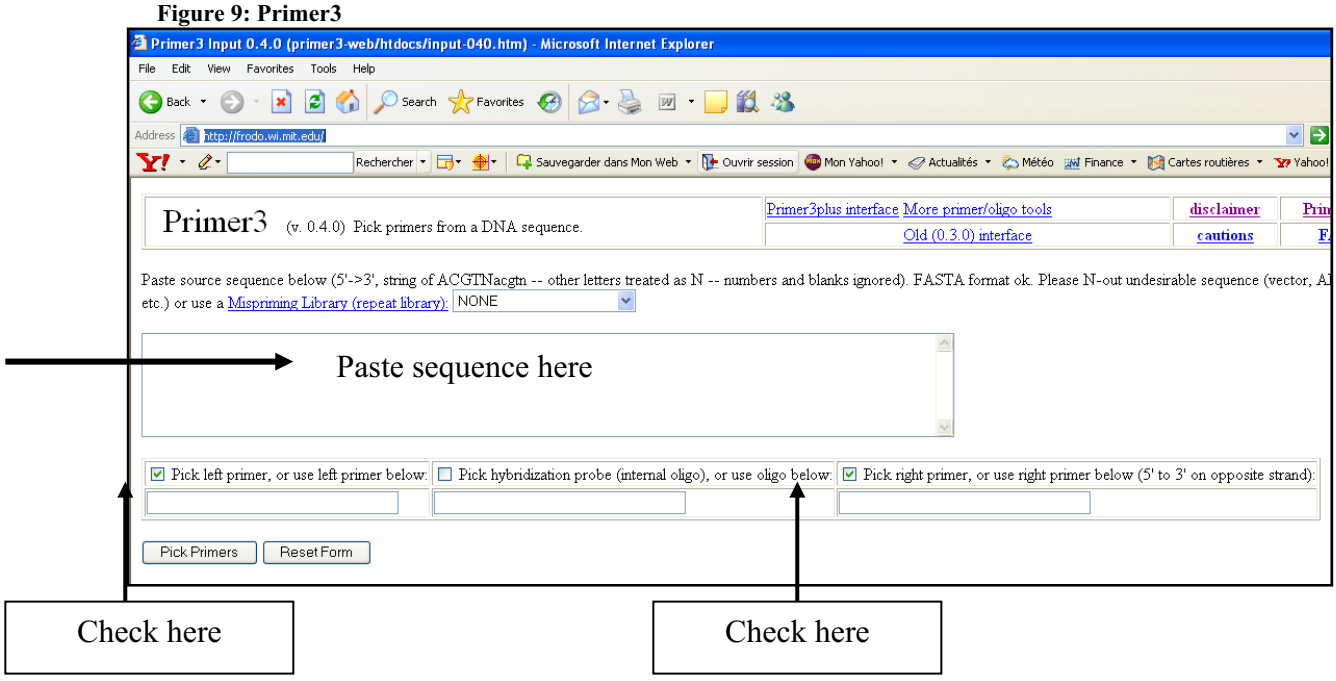
Materials

1. Computer with internet access

Protocol

2. Find the cDNA sequence of the desired gene (by doing a PubMed seach, using NCBI, etc)
3. Copy/Paste the sequence into the appropriate box on the webpage given below and check the appropriate boxes as indicated in
4. Figure 9:
5. http://frodo.wi.mit.edu/

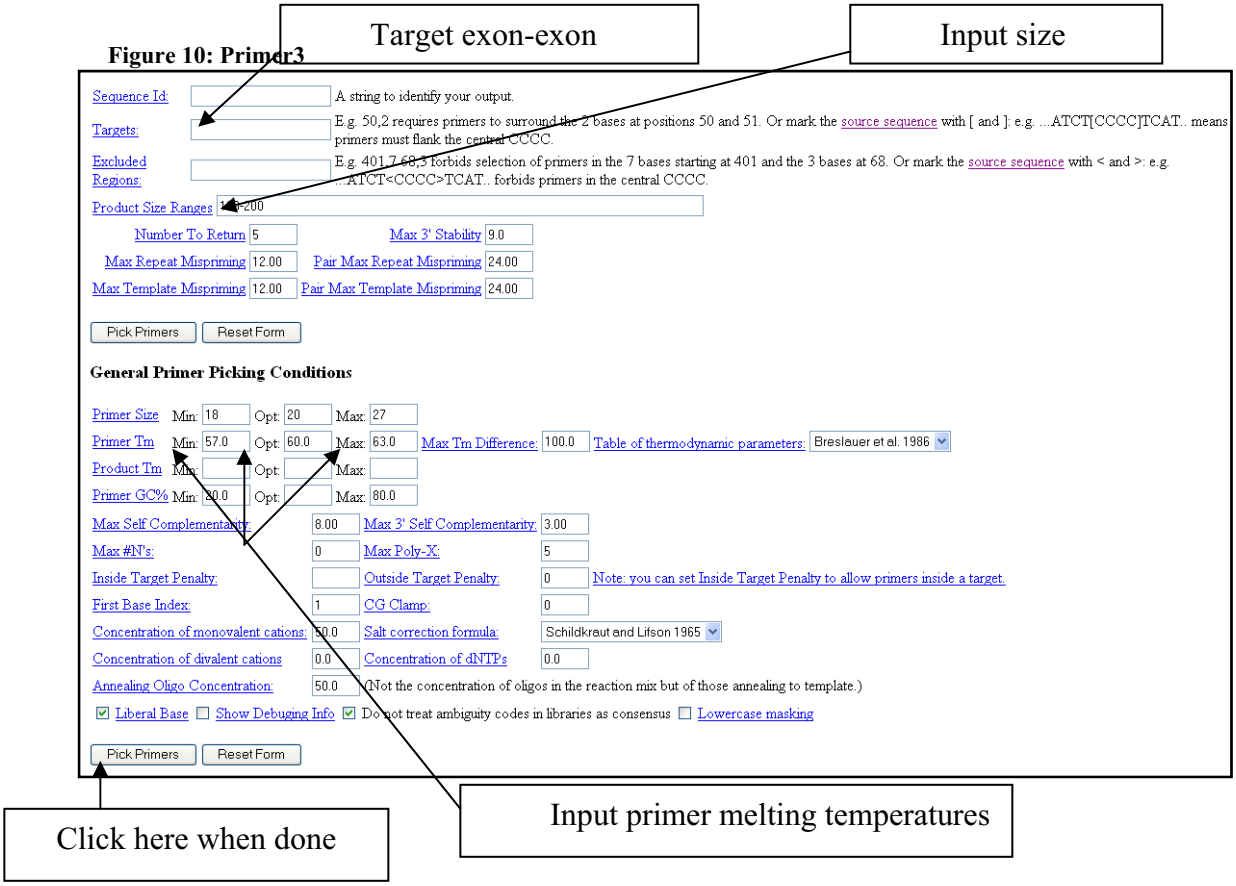
Figure 9: Primer3



6. Take note of exon-exon junctions in cDNA sequence, and use the target box shown in
7. Figure 10 to design primers that span exons
8. Choose a length of PCR product suitable for the final application, and fill in appropriate box on website as indicated in
9. Figure 10:
10. qRT-PCR: amplicon size of 100-200 base pairs
11. Northern: amplicon size of 600-700 base pairs

12. When choosing primers, it is desirable to have a sequence with a GC% around 50% for effective PCR amplification. This corresponds to a primer melting temperature in the range of 57 – 63 °C. Input these values as indicated in
13. Figure 10
14. Click on “Pick Primers” button as indicated in
15. Figure 10 below

Figure 10: Primer3



16. Take note of primer sequence, melting temperature and location (as well as any other desirable information) as determined by the website and order primers.

Note: When performing PCR with the primers designed above, use an annealing temperature 3°C below that of the primer melting temperature.

Reverse Transcription

Protocol based on that provided by ABI for High-Capacity cDNA Reverse Transcription Kits

Materials

1. RNA, quantified (See RNA extraction and quantification)
2. High-Capacity cDNA Reverse Transcription (RT) Kit (ABI)
    a. 10X RT Buffer
    b. 25X dNTP Mix
    c. 10X RT Random Primers
    d. MultiScribe™ Reverse Transcriptase
    e. RNase Inhibitor
3. Nuclease-free water
4. Pipettes and filter tips
5. Thermal Cycler
6. 0.2 µL PCR tubes

Protocol

1. Ensure that RNA to be reverse transcribed is between 0.002 and 0.2 µg/µL.
2. Allow kit components and RNA to thaw on ice.
3. Prepare RT master mix as follows (prepare sufficient master mix for a few extra reactions to account for losses and pipette inaccuracies):

Component	**Volume for 1 reaction (µL)**
10X RT Buffer	2.0
25X dNTP MIX	0.8
10X RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free water	3.2
Total for 1 reaction	10.0

4. Mix master mix gently and place on ice.
5. Pipette 10 µL of master mix into 0.2 µL PCR reaction tubes
6. Pipette 10 µL of RNA sample into each tube, mix by pipetting
7. Seal tubes and centrifuge briefly to spin down contents and eliminate bubbles
8. Load tubes into thermal cycler programmed with the following conditions (20 µL reaction volume):

	Step 1	Step 2	Step 3	Step 4
Temperature (^{0}C)	25	37	85	4
Time (min)	10	120	5	10-60

9. Start the reverse transcription.
10. After step 4, store the cDNA tubes at 2-6 ^{0}C for short term storage, or less than -15 ^{0}C for long term storage.

Quantitative Real Time Polymerase Chain Reaction

Based on the protocol provided with the ABI SYBR® Green Master Mix. "Fast" (~ 1 hour) or "Standard" (~ 2.5 hours) PCR can be performed with this system. All three of the sample block, reaction plate and master mix must be either "Fast" or "Standard" for successful amplification.

Materials

1. Applied Biosystems (ABI) 7900HT Fast Real-Time PCR System
2. ABI SYBR® Green Master Mix (Fast or Standard)
3. Reaction Plate (96- or 384-well, Fast or Standard)
4. Optical Adhesive covers
5. Centrifuge with adaptor for 96- or 384-well plates
6. Microcentrifuge
7. 1.5 mL sterile microcentrifuge tubes
8. Nuclease-free water
9. Pipettes and filter tips
10. Vortexer
11. Forward and Reverse primers
12. Sample cDNA

Protocol (Fast 96-well plate)

1. Thaw primers, cDNA and SYBR® Green Master Mix on ice.
2. In a 2 mL centrifuge tube, combine sufficient SYBR® Green Master Mix and forward and reverse primers for the number of reactions desired plus one or two extra in the following ratio:

Component	Volume for 1 20 µL reaction (µL)
SYBR® Green Master Mix	10
Forward Primer (concentration variable)	2.5
Reverse Primer (concentration variable)	2.5

Total Volume	15

3. Add 15 µL to each well. Each sample, standard and control should be run in triplicate.
4. Standard curve: Add 5 µL of cDNA template (from a positive control sample) + RNase-free water to each well designated as a standard curve. The standard curve cDNA concentrations should span 5 orders of magnitude (Ex: in a 20 µL reaction volume, the standard curve concentrations used were 0.0005; 0.005; 0.05; 0.5; 5 ng/µL)
5. Unknowns: Add 5 µL of cDNA template + RNase-free water to each well. The cDNA concentration in a 20 µL reaction volume should be less than or equal to 1 ng/µL (0.1 ng/µL was used for samples).
6. Seal the plate with an optical adhesive cover and mix with a plate shaker.
7. Centrifuge the plate at 3000 rpm for 1 minute to spin down the contents and eliminate any air bubbles
8. Run the reaction plate on the ABI 7900HT Fast Real-Time PCR System within 2 hours after setting up the system with the following settings:

Stage	**Temperature (°C)**	**Time**	**Cycles**
DNA polymerase activation	95	20 seconds	Hold
Denature	95	1 second	40
Anneal/Extend	60	20 seconds	40
Dissociation curve	95	15 seconds	1
Dissociation curve	60	15 seconds	1
Dissociation curve	95	15 seconds	1
Cool down	40	2 minutes	Hold

9. Store the amplified products at 2-6 ^{0}C for short term storage, or less than -15 ^{0}C for long term storage.
10. Each target should be run on an agarose gel at least once to ensure specific amplification of the target.